FORMULATION AND EVALUATION OF LEVOFLOXACIN HEMIHYDRATE MUCOADHESIVE MICROSPHERES FOR ERADICATION OF *HELICOBACTER PYLORI* INFECTION

A Dissertation submitted to

THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY,

CHENNAI- 600 032

In partial fulfilment of the award of the degree of

MASTER OF PHARMACY

IN

Branch-I -- PHARMACEUTICS

Submitted by

Name: RAGHU .J

REG.No. 261610255

Under the Guidance of

Dr. R. SAMBATHKUMAR, M.Pharm., PhD,

DEPARTMENT OF PHARMACEUTICS

J.K.K.NATTRAJA COLLEGE OF PHARMACY

KUMARAPALAYAM – 638183

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CERTIFICATES

EVALUATION CERTIFICATE

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Internal Examiner **External Examiner**

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DECLARATON

I do hereby declared that the dissertation **"FORMULATION AND EVALUATION OF LEVOFLOXACIN HEMIHYDRATE MUCOADHESIVE MICROSPHERES FOR ERADICATION OF *HELICOBACTER PYLORI* INFECTION"** submitted to "**The Tamil Nadu Dr.M.G.R Medical University - Chennai",** for the partial fulfilment of the degree of **Master of Pharmacy** in **Pharmaceutics,** is a bonafide research work has been carried out by me during the academic year 2016-2018, under the guidance and supervision of **Dr. R. Sambathkumar, M.Pharm., PhD,** Professor, Department of Pharmaceutics, J.K.K.Nattraja College of Pharmacy, Kumarapalayam.

I further declare that this work is original and this dissertation has not been submitted previously for the award of any other degree, diploma, associate ship and fellowship or any other similar title. The information furnished in this dissertation is genuine to the best of my knowledge.

Place: Kumarapalayam **Mr. RAGHU .J**

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1. INTRODUCTION

Helicobacter pylori(H. pylori) infection is associated with a wide range of gastrointestinal diseases including gastritis, peptic ulcer and gastric cancer. Although such infection is asymptomatic in most patients, it always induces an inflammatory response in the gastric mucosa that can be seen histologically. The most significan association of H. pylori is with distal gastric cancer, both intestinal and diffuse types, and meta-analysis has shown that infection confers a 2–3 fold increased chance of developing gastric cancer.[1]

The World Health Organization's international agency for research on cancer declared H. pylori a class I carcinogen in 1994, since when many studies have confirmed this association, the most compelling from Asia.

PREVALENCE OF H. PYLORI& RECENT CHANGES

Recent research has consistently shown that the prevalence of H. pylori is declining in the developed world and especially so in children suggesting thatthe infection will die out in due course. This is one reason put forward to suggest that population screen and treat may be unnecessary in these countries. However, this argument takes no account ofethnic groups, the effects of migration and those economically disadvantaged communities where infection rates are often much higher; therefore,a selective approach to screen and treat

might be considered. The importance of local differences in prevalence is, therefore, important, and a number of interesting studies have been reported this year.An excellent review relating to these issues is set out by Mitchell and Katelaris.[2]A number of original studies have focused on children. One study from Iceland[3] studied205 children aged between 7 and 18 years and found only 3.4% to be infected. However, the prevalence was 2.6% among children where both parents were born in a low prevalence country compared to 17% among those where at least one parent had been born in a high prevalence area (P=.026). Seroprevalence in Icelandic adults is 30%-40%.

Studies from Japan have also shown a considerable fall of H. pylori prevalence in childhood. One study from a high GC incidence area found only 85 of 1,765 (4.8%) students aged 13-15 years to be positive,[4]and in another the prevalence in school children aged 12-15 years was 3.1%.[5] Inoue[6] reported that Japanese generations born before 1950 have a high prevalence of around 80%-90%, decreasing with age to reach around 10% or less in those born around the 1990s,and less than 2% for those born after year 2000. Similar trends are seen in China where in Hangzhou[7] the positivity rates were 14.8%, 20.2%, and 25.8% in 3-6, 7-11 and 12-17 years age groups respectively,with the overall prevalence decreasing from 21.6% to17.2% between 2007 and 2014. In adults undergoing health checks inurban China,[8] the prevalence fell from 31.9% in the1950-1959 birth cohort down to 20% in those born after 1990. This decrease correlated

with the increase in per capita gross domestic product. The prevalence of H. pylori has also declined in Iran[9] where a meta-analysis estimates an overall prevalence of 54%, with a prevalence of 42% in children and 62% in adults. Initial reports of H. pylori infection from Iran had earlier indicated a prevalence of more than 85%. Prevalence continues to decline in Sweden.[10]In Latvia on the other hand there has been no evidence of a fall in prevalence in children over the last 10 years.[11]

H. PYLORI RELATED DISEASE

Acute infection with H. pylori results in histologically proven gastritis clinically manifested by epigastric fullness, vomiting, soft stools, irritability and "putrid breath" as described by Barry Marshall et al in 1985 while trying to fulfill Kochs postulates with self ingestion of live organisms.

This experiment was repeated in 1987 by Morris and Nicholson with similar results and evidence of chronic gastritis. Although spontaneous clearance may occur, the majority of the patients will develop an asymptomatic chronic state in which there is histologicevidence of gastritis with normal gastric acid production.[12]

Infection with H. pylori has been linked to many disease states but data support a strong association with only a few conditions, which include peptic ulcer disease, gastric adenocarcinoma, and

gastric lymphoma.[13] Other associations including the role in non-ulcer dyspepsia have yet to be confirmed.

Peptic Ulcer Disease (PUD)

H.pylori is clearly associated with both duodenal and gastric ulcers. Patients with H. pylori infection have been shown to have at least a threefold increased risk of developing duodenal ulcers.[14]In addition, approximately 90%-95% of patients with duodenal ulcers and 70%-90% with gastric ulcers are infected with H. pylori.[13,15,16] The most important evidence for a causal association between H. pylori and PUD is that the disease process reverses upon the eradication of the organism. Less than 10% of patients that have received an effective treatment against H. pylori have recurrences compared with morethan 70% of those that only received acid-suppressivetherapy.[17,18]The link between H. pylori and PUD has also been reinforced by studies done in smokers in which a twofold increase in the risk of ulcerative disease disappears after cure of H. pylori infection.[19]The role of H. pylori in gastric ulcers, although not as well studied as in duodenal ulcer disease, is similar to duodenal disease.[17]

Although the exact pathogenesis of PUD remains unclear, the following hypothesis has been proposed. H. pylori causes antral endocrine cells to release somato statin[20,21]which results in postprandial gastrin release. This hypergastrinemic state increases

acid production and predisposesthe host to develop gastric metaplasia. Gastric metaplasiais also enhanced by concomitant risk factors such as smoking, alcohol, non-steroidal anti-inflammatory drugs (NSAID) or H. pylori pathogenic factors such as cagA or vacA genotype. It appears that these two genetic loci are relevant to the clinical consequences of H. pylori infection.

Virtually every patient with PUD is infected with aagA positive strain, and vacA positivity determines the interaction with epithelial cells causing the inflammatory reaction and vacuolization reaction.

Gastric adenocarninoma

Although the incidence of gastric cancer has been declining worldwide since the 1930s, it is still one of the most common human malignancies. Evidence for an association between H. pylori infection and gastric cancer first came from epidemiological studies. The prevalenceof H. pylori infection paralleled that of gastric cancer indifferent populations around the world. There is a three to eightfold increase in the risk of gastric cancer in H. pylori infected patients. In addition, H. pylori infection preceded gastric cancer in other studies.[22,23,24] About half of the malignancies involving the gastric body and antrum are linked to H. pylori infection but tumors arising in the gastroesophageal junction are not associated with this infection.[13]Individuals with infection involving the gastric body have a higher risk than those with infection involving the antrum. These

patients seem to have less dense colonization with H. pylofi and a state of hypochlorhydria as compared with patients with antral involvement.[25]On the other hand,most of the people with H. pylori infection will not develop gastric cancer.

A recently published prospective study from Japan that included 1526 patients followed over an average of eightyears.[26]They found a significantly higher incidence of gastric cancer in the H. pylori positive patients with history of nonulcer dyspepsia, gastric ulcers, and hyperplastic gastric polyps, but not among those with duodenal ulcers.

The pathogenesis of gastric cancer is believed to be different than that of PUD. It has been shown that patients with ulcerative disease actually have a lower incidence of gastric cancer.[26,27] It is known that chronic epithelial injury has a carcinogenic effect in many tissues and is thought to be one of the mechanisms implicated in the development of gastric cancer in patients infected with H. pylori. This organism resides in the gastric mucosa and it causes chronic superficial gastritis. Differences in bacterial virulence and a combination of host factors, such as differences in the immune and reparative responses, may determine the ultimate outcome.[28] Inflammation will induce cell proliferation, mutation and eventually selection of the fittest mutant clone.[13,29]There is also a release of free radicals that can damage DNA nucleotides which will lead to mutations and if left unrepaired can result in metaplasia and

cancer.[13]Finally, in 1994 the World Health Organization declared H. pylori to be a type I carcinogen and a definite cause of cancer in humans.[30]The effect of H. pylori eradication in preventing gastric cancer is still unclear. Some studies have shown regression of preneoplastic changes in patients successfully treated for H. pylori,[31,32] but other studies have failed to show this association.[33,34]

Gastric lymphoma

H. pylori infection appears to lead to development ofgastric lymphoid tissue that is not usually found in normal mucosa. This mucosa-associated lymphoid tissue (MALT) can undergo malignant transformation into a rare lowgrade B cell lymphoma of the stomach. This organism has been found in the majority of patients with this type of lymphoma[35]and what is even more remarkable is that 70% of patients with MALT lymphoma have shown to have a complete regression after successful treatment for H. pylori infection.[36] Patients with large tumors or with deep invasion into the gastric wall are less likely to respond to therapy.[34]Reinfection with H. pylori can cause recurrence or the tumor process.[37]

A causative role of H. pylori in the development of non-Hodgkins lymphoma of the stomach, the most common form of primary gastric lymphoma, has also been suggested.[38]Chronic antigenic stimulation by H. pylori has been proposed as the mechanism.[39]

Role in nonulcer dyspepsia

Nonulcer dyspepsia is defined as the presence of pain or discomfort in the epigastrium, associated with nausea, vomiting, heartburn, early satiety, anorexia and belching, and with no evidence of structural or biochemical abnormalities in the gastric mucosa. The annual prevalence in western countries is approximately 25%, and it accounts for about 5% of office visits.[40]Apossible role of H. pylori in the etiology of this entity has been suspected since the organism was first linked to gastritis. However, current evidence does not seem to support this relationship. Some studies, including metaanalyses, have found a slight benefit in terms of symptomatic relief in patients who have received therapy against H. pylori compared with those treated only with acid suppressive therapy.[41,42] These studies have been found to have methodologic weaknesses in the definition of nonulcer dyspepsia, the regimens used, and the documentation of H. pylori eradication was not well documented. A recently published meta-analysis of seven randomized controlled trials, using combination therapy against H. pylori and with adequate follow-up to assess therapeutic response, did not find a significant trend towards a beneficial effect of therapy.[43]

Role in other diseases

H. pylori has been linked to several other clinical conditions,such as hypertrophic gastropathy, bronchiectasis, rosacea,

chronic urticaria, sudden infant death syndrome and coronary artery disease.[13] Some these associations may not actually represent a causative effect of H. pylori and several confounding factors may be implicated.

DIAGNOSIS

Diagnostic tests for H. pylori infection can be divided into two categories, invasive and noninvasive methods. Invasive tests involve an upper gastrointestinal endoscopy with gastric mucosal biopsy and either rapid urease testing, histology, culture or polymerase chain reaction (PCR) tests. The noninvasive tests include antibody detection, carbon labeled urea breath tests and stool antigen detection. When determining the most appropriate test for a given situation, it is important to consider several factors including:

1) if an endoscopy is planned for any other reasons,

2) is it a follow-up test for a residual infection, and

3) prior history of gastric cancer.

Invasive diagnostic tests

Rapid urease tests

Rapid urease tests are relatively inexpensive assaysbased on the principle that a pH change brought on by ammonia produced by H. pylori urease is detected by the use of an indicator.[44]These tests are

highly specific and moderately sensitive.[45,46]Several different test procedures are commercially available. CLOtest derived from Campylobacter-like organism (Ballard Medical Products, Draper, Utah) employs direct placement of urease specimen on an agar gel. A change in color from yellow to red signifies the presence of H. pylori. Results are obtained about 24 h after tissue placement, although most reactions can be detected within 3-4 h. This test has a sensitivity of 75% to 95% and a specificity of 75% to 100%.[47]Two biopsies are recommended to optimize the interpretation, usually one from the antrum and one from the body of the stomach. Other available tests include PyloriTek (Serim Research Corp., Elkhart, Indiana) which uses a semipermeable membrane through which gaseous ammonia can diffuse, accelerating the reaction to about one hour with similar sensitivity and specificity. Also available is the hpfast (GI Suppl~ Camp Hill, Pennsylvania), the newest test, in which a cell-wall detergent is added to the agar in an attempt to improve test performance but clinical evaluations have demonstrated similar results to the CLO test. The rapid urease tests are based on the presence of adequate numbers of bacteria in the specimen. The sensitivityof these tests can be adversely affected by the recentuse of antibacterial agents or medications that could alter the urease activity, such as proton pump inhibitors (PPI) or bismuth compounds.[46]

ERADICATION FAILURE

The success rate of standardor first-line drug therapy which consisting of amoxicillin, clarithromycin and proton pump inhibitor, is gradually decreasing over the last decade. First-line eradication therapies most commonly used in everyday clinical practice fall considerably short of the 80% intention-to-treat (ITT) eradication rates, that are considered the minimal acceptable levels as recommended in the Maastricht guidelines.[48] The objective of *H. pylori* treatment is to achieve 100% eradication, but till date no therapy achieves 100% eradication rate. Dual, triple and quadruple drug treatment therapy failed to eradicate completely in 5 to 50% of patients.[49,50]

FACTORS RESPONSIBLE FOR FAILURE OF *H. PYLORI* ERADICATION THERAPY

Recent biopsy studies[51-55]confirmsthatafter acquiring *H. pylori*penetrates intothe mucus layer of the stomach and fixes itself with glycolipids and phospholipids of mucus gel. *H. pylori,* then disruptsepithelial layer directly or indirectly by releasingof certaintoxinsand enzymes.[55,56]For effective *H. pylori* eradication, antibiotics need to enter into the gastric mucus layer and maintain an effective concentration for sufficient period of time.

Drugs released from conventional tablets or capsules reside shorter duration of time in stomach. Because of its shorter residence time, conventional tablets and capsules are unable to deliver the antibiotics into the mucous layer for sufficient period of time. This is one of the main reason for failure of *H. pylori* eradication therapy. In order to increase the eradication rate, it is essential to design suitable dosage forms to deliver the antibiotics into the site of infection.[57,58]Non compliance, bacterial resistance, cost of drugs and duration of the treatment also influences the *H. pylori* eradication.[59-61]

Antibiotic resistant *H. pylori* strains developed mostly due to the unavailability of required antibiotic concentration at the site of action for sufficient period of time.[62]It is a potentially serious problemin *H. pylori* eradication therapy.

Conventional tablets and capsules are not delivering the sufficient antibiotic concentration for sufficient period of time in the mucus because of its shorter residence time in the stomach. In order to increase contact time, high doses of antibiotics are commonly prescribed, which causes adverse effects and, also it affects entire microbial flora of the gastro-intestinal tract.[63,64]

DRUG DELIVERY SYSTEMS FOR GASTRIC RETENTION

It is essential to design suitable drug delivery systems to deliver the antibiotics into the mucus layer where *H. pylori* exist. Gastric

residence time of the dosage forms is important for delivery of drug into the mucus. Gastroretentive systems are commonly used to increase gastric residence time of dosage forms. Some of the gastroretentive dosage forms discussed below

a. Floating Systems[65,66]

Floating systems were mostly used to increase the gastric residence time of the dosage since1970. Various types of floating systems have been reported, such as hollow microspheres, raft-forming systems, hydrodynamically balanced systems (HBS) and gas-generating systems. Due to the variability in gastric transit times from between person to person, floating systems were not able to produce reproducible gastric residence time, and also these systems required sufficient amount of gastric fluid to allow the systems to float.

b. Mucoadhesion

Fixing of two surfaces is called adhesion. Adhesion of natural or synthetic substances into the biological material are called "Bioadhesion". If the biological material is mucus the term "Mucoadhesion" is commonly used.[67,68]

c. Mucoadhesive Systems

Mucoadhesive systems are adhere into the mucus layer. When the dosage forms deliver the drug at the site of action for prolonged

period, usually the efficacy and bioavailability of the drug is increased.[68]Mucoadhesive systems adhere into the gastric mucus layer for prolonged period, and deliver the drug for sufficiently for longer period of time. Mucoadhesive drug delivery systems are highly suitable for the treatment of *H. pylori*infection, because it can deliver antibioticsdirectly into site of action.

MUCOADHESIVE DRUG DELIVERY SYSTEMS

Mucoadhesive dosage forms adhere into the mucus layer and release the drug at a controlled rate. Various theories have been proposed to explain the mechanisms involved in bioadhesion and mucoadhesion.[69,70]

a. *Mucus: structure, function and composition*

Mucus is a viscous fluid secreted by goblet cells of the stomach. Mucus protects and hydrates the epithelial layer and also it prevents the entry of pathogens and toxic substances into the blood circulation.[71]

b. Composition of mucus

Glycoproteins, lipids, electrolytes and water are the main constituents of mucus.[72]The exact composition of mucus is given below:

1. Water: 95%

2. Glycoproteins and lipids: 0.5–5%

3. Mineral salts: 0.5–1%

4. Free proteins: 1%.

Depending on its site of secretion and certain disease conditions, the composition of the mucus may vary.[71]

c. *Mucin: the glycoprotein of mucus*

Glycoprotein part of the mucus is called mucin. Two forms of mucin are commonly found in mucus, such as membrane bound mucin and soluble secretory mucin.[73-76]Due to its high molecular weight and disulfide bridge, secretory mucins form viscous gels. Membrane-bound mucins contain a hydrophobic domain anchoring the molecules in the plasma membrane. In epithelial surfaces both types of mucins are foundand to protect the surface.

Mucin consists of peptide core (10–30%) and oligosaccharide chains (70–80%). Both are linked linked via o-glycosidic bonds.[77-85]The mucin peptide corecontains high levels ofalanine, serine,glycine, threonine, proline and aromatic amino acids.[86-89]

Oligosaccharide part of the mucus consists of N-acetylglucosamine, galactose, N-acetylgalactosamine, fucose and sialic acid.[89]Mucus exhibit negative charge due to presence of sialic acid and sulfate residues.[90]

d.*Thickness of the mucous layer and its turnover*

The thickness of mucus layer controls the rate of drug entry into the blood circulation. The thickness of human stomach mucous layer has been reported to be 576±81 μm.[91] In general, the thickness of mucus layer varies depending on its site of secretion and, thickness which varies between 50 and 450 μm.[92,93]

Mucus is constantly released by goblet cells and adheres into the epithelial layer for specified period. Mucus is consistently removed from the epithelial layer by peristaltic forces. Turnover time of mucus has not been reported accurately, and usually it varies between 4–6 hours.[94-96]

THEORIES OF MUCOADHESION

There are four main theories that explains the possible mechanisms of mucoadhesion they are given below

1. Electronic theory

2. Adsorption theory

3.Wetting theory

4. Diffusion theory.

a. The electronic theory

According to this theory mucoadhesion occurs due to transfer of electrons between mucoadhesive polymer and mucus.[97,98]

b. The adsorption theory

According to the adsorption theory[99-103]mucoadhesion occurs due to the formation of molecular bonding between mucoadhesive polymer and mucus by van der Waals forces and hydrogen bonds.

c. The wetting theory

The wetting theory[104-108]correlatesthe surface tension of the mucoadhesive polymer and the mucus.

d. The diffusion theory

According to this theory, mucoadhesiveness is achieved by interpenetration of polymer chains of mucus and mucoadhesive polymers.[109-114]

In addition to above motioned theories various polymer structure related and functional groups related factors contribute to varying degrees of polymer/mucus interactions.

FACTORS AFFECTING MUCOADHESION

a. Functional group contribution

Mucoadhesiveness mainly occurs due to interpenetration of polymer chains of mucus and mucoadhesive polymers and, formation of secondary bonding between mucus and mucoadhesive polymers. Secondary non-covalent bonding forms mainly due to hydrogen bond formation between mucus and hydrophilic functional groups of the mucoadhesive polymers such as hydroxyl (OH), carboxyl (COOH), sulphate groups (SO_4H) and amide (NH_2) groups. Polymers that have above motioned functional groups form high number of hydrogen bonds with mucus, andinteract more strongly with mucus.[145]

b. Degree of hydration

Optimal hydration of mucoadhesive polymers is essential for effective mucoadhesion. Hydration of the mucoadhesive polymers occurs due to combination of osmotic forces and capillary action between the mucoadhesive polymer and the mucus layer.[116]Hydration of polymer helps for relaxation of polymer chains and interpenetration of polymer chains. Excess hydration affects mucoadhesion due to the formation of a greasy mucilage.[117]

c. Polymerchain length, molecular weight and degree of cross-linking

Mucoadhesive nature of the mucoadhesive polymers varies depending upon its molecular weight. High molecular weight is necessary for effective mucoadhesion; however, polymer which has extremely long polymer chains,was unable to diffuse and interpenetrate into mucosal surfaces.[118-121]

d. pH and charge

pH value of the physiological environment also influences the mucoadhesive nature of the polymer.[122,123]Mucoadhesive nature of polyacrylic polymers are affected considerably by pH value of the physiological environment. Carboxylic groups of polyacrylic polymers are essential for mucoadhesion. At low pH, these carboxylic groups are available in unionized state and form strong hydrogen bonding with mucus. At elevated pH values, carboxylic groups ionize, unable to form hydrogen bond with mucus. Chitosan, a positively charged polymer, it forms polyelectrolyte complexes with negatively charged mucins and exhibits strong mucoadhesion at high pH value.[124]

e. Polymer concentration

Concentration of the polymer is also considerably affects the strength of mucoadhesive nature of the polymer. The optimum

polymer concentration is varies depending upon the physical state of the dosage form.[125]

MUCOADHESIVE POLYMERS

The mucoadhesive polymers that are commonly used in the preparation of mucoadhesive dosage forms are commonly classified into two types.

1. First generation mucoadhesive polymers
2. Second generation mucoadhesive polymes

a. First generation mucoadhesive polymers

The firstgeneration mucoadhesive polymers are subdivided into three categories:

(1). Anionic polymers

(2). Cationic polymers and

(3). Non-ionic polymers

- ***Anionic polymers***

For the preparation of pharmaceutical formulations,anionic polymers are most widely used, because of its low toxicity and high mucoadhesive nature. Polymers which have carboxyl and sulphate functional groups are called anionic polymers. The most widely used anionic polymer is poly(-acrylic acid) (PAA).It has excellent mucoadhesive nature, due to the formation of strong hydrogen bonding with mucus[126].PAA are non-toxic, non-irritant and considered

safe (GRAS (Generally Recognized As Safe) status) for oral use by the FDA.[127,128]

- ***Cationic polymers***

Chitosan is the most widely used cationic polymer. Chitosan is produced by the deacetylation of chitin. Chitosan is mostly preferred because of its polysaccharide nature, biodegradability,biodegradability and less toxic nature.[129]Chitosan binds with mucus by ionic interactions mechanism. It interacts with sialic acid and sulphonic acid substructures of mucus. Moreover, the amino groups and hydroxyl also interact with mucus by hydrogen bonding.[129]

- ***Non-ionic polymers.***

Hydroxypropylmethyl cellulose (HPMC) and Methyl cellulose (MC) are commonly used nonionic polymers. Non-ionic polymers have less mucoadhesive property compared to polyelectrolytes because of its weak interactive nature with mucus.[129] Mucoadhesive property of non ionic polymers are mainly occurs due to the penetration of its polymer chains into the mucus.[130]

b. Second generation mucoadhesive polymers

- ***Lectins***

Lectinsare made up of proteins and glycoproteins. It binds with carbohydrate molecules of epithelial cells reversibly.After binding with

cells, the lectins can either remain present on the cell surface or get engulfed via a process of endocytosis. Because of this nature, lectins are used to target the drug. Some bacteria use lectins to fix with the cells of the host during infection. Lectins are not commonly used because of its immunogenic or anaphylaxis nature.[131,132]

- ***Bacterial adhesions***

K99-fimbriae, an attachment lectin, obtained from *E. coli*is most widelyused to target the drug into gastrointestinal tract, and also it covalently attaches with polyacrylic acids.[133]

Recently, a new types of mucoadhesive polymer has been introduced into the market. These new types of mucoadhesive polymers are prepared by introducing thiol groups into the polymeric backbone of established mucoadhesive polymers. Thiol groups interact strongly with cysteine rich port of mucus by forming disulfide bonds.[133]These disulfide bonds are not affected by ionic strength and pH of the physiological environment.

Example of thiolated polymers.[134]

- Poly(acrylic acid)–homocysteine
- Chitosan–iminothiolane
- Poly(methacrylic acid)–cysteine
- Chitosan–thioethylamidine
- Poly(acrylic acid)–cysteine

- Chitosan–thioglycolic acid
- Sodium carboxymethylcellulose–cysteine
- Poly(acrylic acid)–homocysteine
- Alginate–cysteine

MUCOADHESIVE SYSTEMS IN ORAL DRUG DELIVERY

Oral mucoadhesive drug delivery systems extend the residence time of dosage forms in gastric or small intestine. Mucoadhesive systems, commonly used to deliver the drug into the site of action, target the drug into certain parts of GI tract and prolong the drug delivery.

A number of mucoadhesive dosage forms, including nanoparticles, semisolid dosage forms, microspheres, powders, sustained release tablets have been widely reported.

a. Mucoadhesive microspheres

Microsphere plays an important role in particulate drug delivery systems,because of its size and its good carrier property. One of the main drawbacks of microspheres is by its shorter gastric residence nature. These drawbackshave now been solved by coupling the mucoadhesive property to the conventional microspheres, by preparing novel “Mucoadhesive microspheres.”

Mucoadhesive microspheres are commonly prepared by using mucoadhesive polymers or coating of conventional microspheres with mucoadhesive polymers. The size of the mucoadhesive polymers commonly varies between 1–1000 μm.[135] Mucoadhesive microspheres can be tailored to stay to any mucosal tissue including those found in urinary tract, GI tract, nasal cavity and eye.

2. DRUG PROFILE[136]

Levofloxacin hemihydrate

Generic and additional names Levofloxacin hemihydrate

Synonyms BAY 12-8039

Molecular formula C18H20FN3O4, ½ H2O

Molecular weight 370.4

Description LFX is a yellowish white to yellow powder

Solubility Freely soluble in glacial acetic acid, chloroform; sparingly soluble in water

Melting point 214- 216°C

Category Anti-Bacterial Agents, Anti-Infective Agents, Quinolones

Phamacokinetics

- Absorption LFX is rapidly and entirely absorbed after oral dose.
- bioavailability 99%
- Protein Binding 24 – 38 %
- Excretion Urinary
- Plasma Half Life 6 to 8 hr

Mechanism of action

LFX is L form of the racemate, OFX, a quinolone antimicrobial agent. The antibacterial activity of OFX resides primarily in L-isomer. The MOA of LFX involves destroying of bacterial topoisomerase and di-nucleotide adenosine gyrase enzymes required for di-nucleotide adenosine replication, transcription, repair and recombination. LFX

exhibits in vitro MIC of two mcg/mL or less against most (•90%) strains

Dose (*H.Pylori* infection)[137]

Levofloxacin 500 mg b.d

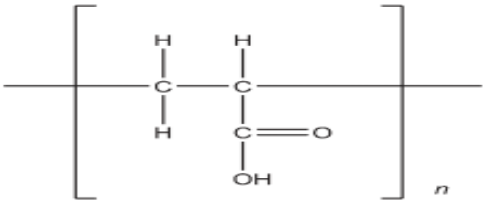
3. POLYMER PROFILE

CARBOPOL 974P[138]

Nonproprietary Names: Carbopola, Carbopols.

Synonyms: Poly acrylic acid polymer, carbopol, acrylic acid, carboxyvinyl polymer, Acritamer, carboxy poly methylene.

Structural Formula



The polymer chains of carbopols are crosslinked with allyl pentaerythritol and polymerized in ethyl acetate.

Description: Carbopols are white-colored, hygroscopic, fluffy powders

Molecular Weight: 12000-140000

Melting point: Decomposition occurs at 260ºC.

Glass transition temperature:100 –105°C.

Moisture content: Normal content is up to 2%.

Solubility: Carbopol 974p insoluble in water, dilute acids, and common organic solvents.

Stability: Stable though hygroscopic and can be heated at temperatures below 104ºC up to 2 hours without affecting their thickening efficiency.

Safety: Carbopols are generally nontoxic and nonirritant. There is no hypersensitivity reactions are reported.

HYDROXYPROPYLMETHYLCELLULOSE (HPMC)[139]

Non-proprietary Names: Hypromellose (BP), Hydroxypropyl methyl cellulose (JP), Hypromellosum (PhEur), Hypromellose (USP).

Synonym: Tylopur, Benecel MHPC, Metolose, E464, hydroxypropyl methylcellulose, Methocel, methylydroxypropylcellulose HPMC.

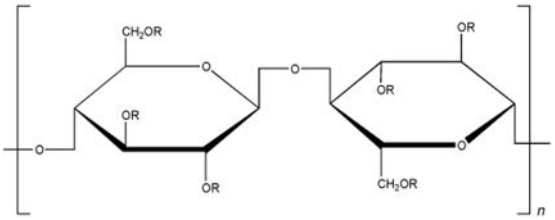
Chemical Name:[C6H7O2(OH)z(OCH3)x(OCH2CHOHCH3)y]n

Description: Odorless and tasteless white creamy powder.

Molecular Weight:10000-1500000.

Grades: Methocel K100 Premium LVEP, Metolose 90SH, Methocel K4M Premium, Metolose 65SH, Methocel K15M Premium, Metolose 60SH, Methocel K100M, Methocel E50 Premium LV, Premium, Methocel E4M Premium, Methocel E15 Premium LV, Methocel F50, Methocel E6 Premium LV, Premium Methocel E10M Premium CR, Methocel E5 Premium LV, Methocel E3 Premium LV.

Structural formula



Where R is H, CH3, or CH3CH (OH) CH2

Glass transition temperature:170 - 180°C.

Moisture content:HPMC absorbs moisture from the atmosphere.

Solubility: Soluble in cold water, practically insoluble in ethanol (95%), chloroform and ether.

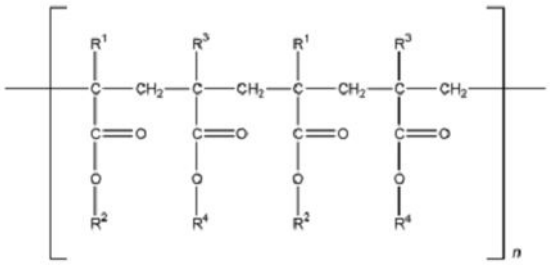
Viscosity:HPMC K4M having viscosity of 4000 cps (2% w/v, at 20°C)

EUDRAGIT RS 100[140]

Synonyms: Polymeric methacrylates, Eastacryl 30D, Kollicoat MAE 30 D, Acryl-EZE, Kollicoat MAE 30 DP, Eudragit.

Chemical name: Poly (ethyl acrylate, methyl methacrylate, trimethylammonioethyl methacrylate chloride) 1: 2: 0.1

Structural Formula



Eudragit RS 100: R1=H, CH3;R2=CH3 ,C2 H5;R3=CH3;R4=CH2CH2 N(CH3)3+Cl-

Solubility: Soluble in acetone and dichloromethane. Insoluble in petroleum ether and water.

Density (bulk):0.390 g/cm3

Density (tapped):0.424 g/cm3

Refractive index:1.38–1.385 for Eudragit RL and RS

Safety: Polymethacrylate copolymers generally regarded as nontoxic and nonirritant materials.

4. LITERATURE REVIEW

Umamaheshwari et al., (2002)[141] prepared acetohydroxamic acid entrapped floating mucoadhesive microspheres by emulsion solvent diffusion technique. A 2% (w/v) solution of polycarbophil was used to prepare floating mucoadhesive microspheres. In vitro floating studies, detachment force and in vivo studies were confirmed the potential of these microspheres.

Umamaheshwari et al., (2003)[142] developed acetohydroxamic acid loaded polycarbonate microballoons by a solvent evaporation method. In simulated gastric fluid In vitro release studies were conducted. About 74% to 85% of microballoons were floated up to 12 h. In vitro cell growth studies were conducted by using H. pylori culture and in vivo studies were conducted by using H. pylori infected Mongolian gerbils. Prepared microballoons demonstrated 10 times higher anti-H. pylori action when compared to plain acetohydroxamic acid solution.

Hejazi and Amiji (2003)[143] developed tetracycline loaded chitosan microspheres for H. pylori infection. Suspensions of prepared microspheres were given to gerbils. Gerbils were killed at different time intervals to assess the radioactivity in gastric fluids and tissues. 11% of chitosan microspheres remained in the stomach after 10 hours of administration. Higher tetracycline concentration was observed in

the stomach than plain drug solution and non crosslinked microspheres.

Schicho Higo et al., (2004)[144] developed tetracycline loaded sucralfate acidic complex for eradication of H. pylori. In vitro results confirmed that more amount of tetracycline loaded sucralfate acidic complex retained on the gastric mucosa than physical mixture of tetracycline and sucralfate. Addition of acid during the formulation dissociated the aluminium hydroxide groups from the binding sites and produced more binding sites.

Amiji (2004)[145] developed tetracycline loaded chitosan microspheres. Efficacy of prepared microspheres was evaluated by using the fasted Mongolian gerbils. Tetracycline loaded chitosan microspheres were given to H. pylori infection induced gerbils. A considerable increase in H. pylori eradication activity was observed in comparison to aqueous solution of the drug.

Ishak et al., (2007)[146]prepared metronidazole beads using chitosan and alginate by ionotropic gelation method. Prepared beads showed optimum drug entrapment efficiency, immediate buoyancy, and extensive drug release profile. In vivo H. pylori studies showed that metronidazole (dose 15 mg/kg) floating beads provided 100% H.

pylori clearance while the metronidazole suspension (dose 20 mg/kg) provided only 33.33% clearance.

Rajinikanth et al., (2008)[147] developed floating gel system of acetohydroxamic acid. Prepared floating systems formed gel immediately and floated for longer period time in simulated gastric fluid (pH 1.2). In vivo studies confirmed the anti H. pylori activity of floating gel system in gerbil model. Authors concluded that the quantity of acetohydroxamic acid required for H. pylori eradication effect was very less in floating gel system than acetohydroxamic acid suspension.

Tan S et al., (2009)[148]Real-time electronic speckle pattern interferometry method has been applied to study the diffusion behavior of levofloxacin mesylate (MSALVFX) in agarose hydrogel. The results show that the diffusivity of solute decreases with the increase of concentration of agarose and adapts to Kohlrausch's law. Furthermore, Amsden's model, based on the retardance effect associated with polymer chain flexibility, was employed to simulate the diffusion behavior. The consistent results suggest that the retardance effect dominates the diffusion process of MSALFVX in hydrogel; moreover, polymer chain flexibility greatly affects drug transport within the polymer matrix.

Chang et al., (2010)[149] prepared berberine nanoparticles for the eradication of H. pylori. Chitosan was used to prepare nanoparticles. The effect of the nanoparticles and their mechanisms were evaluated by using human gastric carcinoma epithelial cell line. The prepared nanoparticles significantly suppressed the H. pylori growth and reduced cytotoxic effects of H. pylori.

Arora and Budhiraja (2011)[150] prepared floating metronidazole tablets for H. pylori eradication. Tablets were prepared by using carbopol 971P and methocel K100LV. The floatability and drug release increased in the presence of sodium bicarbonate, microcrystalline cellulose and sodium citrate. The optimized formulation provided drug release up to 12 hours by anomalous diffusion mechanism.

Vasilev K et al., (2011)[151] prepared plasma polymerization of n-heptylamine for the generation of two thin coated layers that serve two distinct purposes. First, an n-heptylamine plasma polymer layer is applied onto the surface of the solid carrier material in order to facilitate spreading of the drug, which is applied by solvent casting; levofloxacin in ethanol was used for this study. A second n-heptylamine plasma polymer coating then serves as a thin barrier coating to control the release. We show that the rate of release can be adjusted via the thickness of the plasma polymer overlayer. We also show that this modality of controlled release of levofloxacin completely

inhibits Methicillin-resistant Staphylococcus aureus (MRSA) colonization and biofilm formation on and near the coated biomaterial surface.

Kumar G et al.,(2012)[152] prepared novel poly(lactic-co-glycolic acid) (PLGA)-based nanoformulation of levofloxacin for multidrug-resistant tuberculosis with the purpose of achieving sustained release in plasma. After lyophilization of levofloxacin-loaded nanoparticles, the average size, charge, and polydispersity index were 268 ± 18 nm, -10.2 ± 1.5 mV, and 0.15 ± 0.03, respectively. The maximum drug encapsulation efficiency and loading capacity were 36.9 ± 6.1% (w/w) and 7.2 ± 1.2 mg/100 mg nanopowder, respectively. Biphasic extended-release profile was produced in vitro. Scanning electron microscopy and Fourier transform infrared studies showed spherical shape of drug-loaded nanoparticles and no drug-polymer interactions were observed. After single oral administration in mice, levofloxacin-loaded PLGA nanoparticles produced sustained release of levofloxacin for 4 days in plasma against 24 h for free levofloxacin. Levofloxacin was detected in organs (lung, liver, and spleen) for up to 4-6 days in case of levofloxacin-loaded nanoparticles, whereas free levofloxacin was cleared within 24 h. This novel formulation did not show any significant adverse effects on body weight and clinical signs in mice. No treatment-related changes were found in hematological and biochemical parameters and on histopathological evaluation. These

results indicate the feasibility of development of an orally efficacious safe formulation of levofloxacin with sustained-release properties.

El-Zahaby SA et al., (2014)[153] prepared gastroretentive levofloxacin (LVF) floating mini-tablets for the eradication of Helicobacter pylori (H. pylori). They were prepared using the matrix forming polymer hydroxypropyl methylcellulose (HPMC K100M), alone or with Carbopol 940P in different ratios by wet granulation technique. Buoyancy of mini-tablets was achieved by an addition of an effervescent mixture consisting of sodium bicarbonate and anhydrous citric acid to some formulations. The prepared mini-tablets were evaluated for weight variation, thickness, friability, hardness, drug content, in vitro buoyancy, water uptake and in vitro release. The optimized formula was subjected to further studies: FT-IR, DSC analysis and in vivo examination in healthy volunteers. The prepared mini-tablets exhibited satisfactory physicochemical characteristics. Incorporation of gas-generating agent improved the floating parameters. HPMC K100M mini-tablet formulation (F1) offered the best controlled drug release (>8 h) along with floating lag time <1 s and total floating time >24 h. The obtained DSC thermograms and FT-IR charts indicated that there is no positive evidence for the interaction between LVF and ingredients of the optimized formula. The in vivo test confirmed the success of the optimized formula F1 in being retained in the stomach of the volunteers for more than 4 h. LVF

floating mini-tablets based on HPMC K100M is a promising formulation for eradication of H. pylori.

El-Zahaby SA et al., (2014)[154] prepared size increasing (plug-type) levofloxacin hemihydrate (LVF) tablets for eradication of Helicobacter pylori (H. pylori). They were prepared using in situ gel forming polymers including: gellan gum, sodium alginate, pectin and xanthan gum. Effect of cross-linkers: calcium and aluminum chloride, on the drug release was also studied. The prepared tablets were evaluated for their physicochemical parameters: weight variation, thickness, friability, hardness, drug content, water uptake and in vitro drug release. The optimized formula was subjected to further studies such as radial swelling test, FT-IR and DSC. Results revealed that LVF release depends not only on the nature of the matrix but also on the type of cross linker used to form this polymeric matrix. The addition of either calcium chloride or aluminum chloride, as cross-linkers, to gellan gum formulations significantly decreased drug release. Other polymers' formulations resulted in increased drug release upon addition of the same cross-linkers. The formula containing xanthan gum without any cross linker showed the most sustained LVF release with an increase in diameter with time, thus acting as a plug-type dosage form. IR spectra and DSC thermograms of LVF, xanthan gum, and a physical mixture of both, indicated that there was no

interaction between the drug and the polymer and confirmed the drug stability.

Merchant Z et al., (2014)[155]prepared powders by spray-drying from an aqueous solution containing levofloxacin and chitosan/amphiphilic octanoyl chitosan. l-leucine was also used to assess its effect on aerosolization. Following spray-drying, the resultant powders were characterized using scanning electron microscopy, laser diffraction, dynamic light scattering, HPLC, differential scanning calorimetry, thermogravimetric analysis and X-ray powder diffraction. The in vitro aerosolization profile was determined using a Next Generation Impactor, whilst in vitro antimicrobial assessment was performed using MIC assay. Microparticles of chitosan have the property of mucoadhesion leading to potential increased residence time in the pulmonary mucus, making it important to test the toxicity of these formulations. In-vitro cytotoxicity evaluation using MTT assay was performed on A549 cell line to determine the toxicity of formulations and hence feasibility of use. The MTT assay confirmed that the polymers and the formulations were non-cytotoxic. Hydrophobically modifying chitosan showed significantly lower MIC (4-fold) than the commercial chitosan against P. aeruginosa. The powders generated were of suitable aerodynamic size for inhalation having a mass median aerodynamic diameter less than 4.5µm for formulations containing octanoyl chitosan. These highly dispersible powders have minimal moisture adsorption and

hence an emitted dose of more than 90% and a fine particle fraction (FPF) of 52%. Powders with non-modified chitosan showed lower dispersibility, with an emitted dose of 72% and FPF of 20%, as a result of high moisture adsorption onto the chitosan matrix leading to cohesiveness and subsequently decreased dispersibility.

Jalvandi J et al., (2017)[156] prepared a range of biodegradable drug-nanofibres composite mats drug delivery systems. The results showed that controlled release of levofloxacin (LVF) could be achieved by covalently binding LVF to low molecular weight chitosan (CS) via a cleavable amide bond and then blending the conjugated CS with polyvinyl alcohol (PVA) nanofibres prior to electrospinning. PVA/LVF and PVA-CS/LVF nanofibres were fabricated as controls. The conjugated CS-LVF was characterized by FTIR, DSC, TGA and 1H NMR. Scanning electron microscopy (SEM) showed that the blended CS-PVA nanofibres had a reduced fibre diameter compared to the controls. Drug release profiles showed that burst release was decreased from 90% in the control PVA/LVF electrospun mats to 27% in the PVA/conjugated CS-LVF mats after 8h in phosphate buffer at 37°C. This slower release is due to the cleavable bond between LVF and CS that slowly hydrolysed over time at neutral pH. The results indicate that conjugation of the drug to the polymer backbone is an effective way of minimizing burst release behaviour and achieving sustained release of the drug, LVF.

Zhang LP et al., (2018)[157] prepared liquid crystalline molecularly imprinted polymers (LC-MIPs) by low cross-linking. The multiwalled carbon nanotubes (MWCNTs) coated LC-MIP (MWCNT@LC-MIP) was the first fabricated as a novel floating interaction-controlled DDS. The synthesis was achieved by adding 9-vinylanthracene to obtain the high-density vinyl group functionalized MWCNTs firstly, and then polymerization of LC MIPs was performed on the surface of MWCNTs using a mixture of methacrylic acid, ethylene glycol dimethacrylate, and 4-methyl phenyl dicyclohexyl ethylene (LC monomer) with levofloxacin (LVF) as model template drug. Both template/functional monomer ratio and levels of crosslinker were optimized to obtain the best imprinting factor. Characterizations of polymer were investigated by the transmission electron microscope, nitrogen adsorption, thermogravimetric analysis. The release profiles showed an obvious zero-order release of LVF from MWCNT@LC-MIP, which exhibited 3.8 μg/h of the release rate with duration of about 20 h. In vivo pharmacokinetic study displayed the relative bioavailability of the gastro-floating MWCNT@LC-MIP was 578.9%, whereas only 58.0% of MWCNT@MIP and 11.7% of the bared MWCNT. As a conclusion, MWCNT@LC-MIP showed potentials for oral administration by the innovative combination of floating and controlled release properties.

5. AIM AND OBJECTIVES OF THE WORK

Helicobacter pylori (H. pylori) causes chronic gastritis, peptic ulcer, gastric cancer and gastric MALT lymphoma. Guidelines support treatment irrespective of symptoms and complications. Success rates of empirical therapies have fallen in recent years in many countries.The "key" antibiotics in the treatment of *H. pylori* infection are clarithromycin and levofloxacin. After failure of an empirical first-line treatment, physicians use a levofloxacin tripletherapy (PPI + levofloxacin + amoxicillin) or a bismuth quadruple therapy (6-8). Inparticular, levofloxacin triple therapy is the treatment of choice after failure ofbismuth quadruple therapy. Even though, treatment fails to eradicate *H. pylori* infection completely. The main reasons given for the treatment failure are the short residence time of antimicrobial agents in the stomach and availability of insufficient antimicrobial concentration in the mucus layer of the stomach where *H. pylori*resides, emergence of antibiotic-resistant strains and poor adherence possible due to complicated regimens and drug-related side-effects.

It is therefore, essential to design suitable dosage forms that not only solve the limitations of conventional delivery systems but also deliver the antibiotics to the site of action. To improve treatment of *H. pylori* infection, by achieving required bactericidal concentrations of antibiotics in the stomach, it is assumed that the novel formulations

adhering to the mucus layer and releasing the drug at the site of infection would be significantly more effective than conventional dosage forms. Mucoadhesive drug delivery systems may prolong the gastric residence time of the antibiotics because they adhere to the mucus and also deliver the antibiotics directly into the mucus where H. pylori exists. Among the mucoadhesive drug carriers, mucoadhesive microspheres have some advantages because of its close contact with the mucus, lightweight and smaller dose variation. Hence, in this study, mucoadhesive microsphere drug delivery system was selected to deliver levofloxacin effectively into the mucus.

Aim

The aim of the present research work is to formulate and evaluate mucoadhesive microspheres of levofloxacin by using the ucoadhesive polymers for H. pylori eradication.

Objectives of the present work is to

1. Deliver the antibiotics at the site of infection.
2. Prolong the delivery of the antibiotics at the site of infection.
3. Minimize the dosing frequency.
4. Reduce the amount of drug required for *H. pylori* eradication.
5. Minimize of side effects associated with levofloxacin.
6. Reduce the duration of treatment.
7. Develop optimized dosage form to improve patient compliance.

To achieve the above therapeutic needs effectively, the drug delivery system should have mucoadhesive and extended release property. To achieve the mucoadhesive and extended release property in this study, combination of mucoadhesive polymers, such as carbopol 974P and Hydroxy Propylmethyl cellulose K4M (HPMC K4M) were used. Eudragit RS 100 used as matrix polymer.

6. MATERIALS AND INSTRUMENTS

a. Materials

S.No	Materials	Supplier
1	Acetone	SD Fine chemicals, Mumbai.
2	Levofloxacin hemihydrate	Goldsun Pharmaceuticals limited, Mumbai.
3	Carbopol 974P	Macleods Pharmaceuticals limited, Mumbai.
4	Eudragit RS100	Macleods Pharmaceuticals limited, Mumbai.
5	HPMC K4M	Macleods Pharmaceuticals limited, Mumbai.
6	Hydrochloric acid	Qualigens fine chemicals, Mumbai.
7	Light liquid paraffin	SD Fine chemicals, Mumbai.
8	Methanol	Merck specialties pvt. Ltd. Mumbai.
9	n-Hexane	SD Fine chemicals, Mumbai.
10	Sodium Hydroxide	SD Fine chemicals, Mumbai.
11	Span 80	SD Fine chemicals, Mumbai.

b. Instruments and apparatus

S.No	Name of the Instrument	Name of the Manufacturer and Model
1	Differential scanning calorimeter	Universal Instruments. (Model: V4.2E TA)
2	Digital balance	Shimadzu Scientific Instruments. (Model: BL-220H)
3	Dissolution test apparatus	Labindia. (Model: Disso 2000)
4	FTIR Spectrophotometer	Shimadzu, Japan. (Model:FTIR-84005)
5	Glass wares	Sigma Scientific Glass Pvt. Ltd,Chennai.
6	Laboratory shaker/vibrator	Xi'an Depai Biotechnology Co., Ltd.(Model: BILON-COS-100B)
7	Magnetic stirrer	REMI Laboratory Instruments. (Model: 2 MLH).
8	Mechanical stirrer with Digital rpm display	REMI Laboratory Instruments. (Model: RQ-121/D).
9	Particle size Analyzer	Particle sizing systems, Inc, Santa Barbara, Calif., USA (Model: 780 AccuSizer)
10	Scanning Electron Microscope	Hitachi High-Technologies. (Model: S – 450).
11	Stability chamber	REMI Laboratory Instruments.(Model: Remi CHM-10 S®)
12	Tablet disintegration test machine (I.P. STD. 1985)	Scientechinstruments.Delhi.
13	UV/Visible double beam Spectrophotometer	Shimadzu Analytical (India) Pvt. Ltd.

14	Vacuum dryer	Saga Engineering Co (Model:SO-150)
15	Vortex mixer	Alfa Medical instruments (Model:RX3 Vortex mixer)
16	X-ray powder diffractometer	Bruker AXS. (Model: D8 Advance diffractometer).

7. METHODOLOGY

PREFORMULATION STUDIES

Standard curve of Levofloxacin hemihydratein 0.1 N HCl

Stock solution of Levofloxacin hemihydrate(100 µg/ml) was prepared in 0.1 N HCl, repeated three consecutive days and each day in triplicate to find the inter- and intra-day variations. It was further diluted to obtain the known standard solutions in range of 1-10 µg/ml. Absorbance was measured spectrophotometrically (Shimadzu UV/Visible spectrophotometer 2100; Tokyo, Japan) at 293 nm. The mean data (n=9) were used for the preparation of calibration curve. The concentration of the dissolved drug was calculated from regression equation obtained from calibration curve.

FORMULATION OF MUCOADHESIVE MICROSPHERES

Microspheres were prepared by emulsionsolvent evaporation method.[158,159] Liquid paraffin and acetone were used as solvent system. 0.75% w/v Span80 was used to prevent agglomeration of microspheres during preparation.Weighed quantity ofLevofloxacin hemihydrateand Eudragit RS 100 was dissolved in 30 ml acetone.Carbopol 974P and HPMC K4M were dispersed in it. This dispersion was cooled to 5°C, and added slowly into 300 ml of liquid paraffin, which was previously cooled to 5°C,[160,161]with stirring(700rpm). The resulting emulsion was stirred at 40°C for 40

min. Finally, the suspension of microspheres were filtered and washed by using n-hexane and the prepared microspheres were dried by usingvacuum dryer at room temperature overnight.

Formulation composition of the mucoadhesive microspheres of Levofloxacin hemihydrate

Formulation code	Eudragit RS 100 (% w/v)	Carbopol 974P (% w/v)	HPMC K4M (% w/v)	Levofloxacin hemihydrate (% w/v)
LM1	2	1	1	5
LM2	4	1	1	5
LM3	6	1	1	5
LM4	8	1	1	5
LM5	6	0.5	1	5
LM6	6	1.5	1	5
LM7	6	2	1	5
LM8	6	1	0.5	5
LM9	6	1	1.5	5
LM10	6	1	2	5

EVALUATION OF MUCOADHESIVE MICROSPHERES

Determination of percentage yield of microspheres[162]

Dried microspheres were collected and weighed accurately using a digital balance. The percentage yield of prepared microspheres was calculated by using the formula mentioned below:

$$\text{Percentage yield of microspheres} = \frac{\text{Weight of microspheres of obtained}}{\text{Total weight of drug and polymers}} \times 100$$

Determination of drug content and encapsulation efficiency[163]

The drug content of the microspheres were measured by extraction method. Accurately weighed 5 mg of mucoadhesive microspheres were crushed in to a powder using glass mortar and pestle. The resulting powdered mucoadhesive microspheres were dispersed in 15 ml of 0.03 M sodium hydroxide solution. The final suspension was vortexed at 2500 rpm for 1 minute and then for a further 2 hours at 1000 rpm and room temperature. In order to calculate the amount of drug entrapped in the mucoadhesive microspheres, the suspensionwas then filtered through a 0.45µm syringe filter and the filtrate was analyzed by HPLC.[164]

$$\text{Drug content in microspheres} = \frac{\text{Weight of drug in microspheres}}{\text{Weight of microspheres}} \times 100$$

$$\text{Encapsulation efficiency} = \frac{\text{Actual drug encapsulated}}{\text{Theoretical drug encapsulated}} \times 100$$

Particle size analysis

Particle size of the drug, excipients and prepared microspheres were measured by using laser based particle size analyzer (780 AccuSizer,Particle sizing systems Inc, USA). The particles were dispersed in n-Hexane, and suspended mechanically by magnetic stirring during the analysis.

Shape and surface characterization

The shape and surface characteristics of the microspheres were observed under a Scanning Electron Microscope (SEM). HITACHI-SEM MODEL S – 450 model scanning electron microscope was used for the study. The prepared microspheres were placed directly on to the SEM sample holder by using double-sided fixing tape and coated with gold film (thickness 200 nm) under reduced pressure (0.001 torr) and photographed.

***In vitro* evaluation of mucoadhesiveness**[165]

A periodic acid/Schiff (PAS) colorimetric method reported by Mantle and Allen[166]was used to determine the free mucin concentration in order to assess the amount of mucin adsorbed on the Levofloxacin hemihydrate mucoadhesive microspheres and its effect on the assessment of mucoadhesive behavior of prepared mucoadhesive microspheres.

Two reagents were prepared. Schiff reagent contained 100 mL of 1% basic fuchsin (pararosaniline) aqueoussolution and 20 mL of 1 M HCl. Sodium metabisulphite (0.1g) was added to every 6 mL of Schiff reagent before use, andthe resultant solution was incubated at 37°C until it becamecolorless or pale yellow. Periodic acid reagent was freshlyprepared by adding 10 µL of 50% periodic acid solution to 7mL of 7% (vol/vol) acetic acid solution.Standard calibration curves were

prepared from 2 mL ofmucin standard solutions (0.25, 0.5, 0.75, and 1 mg/2 mL).

After adding 0.2 mL of periodic acid reagent, the sampleswere incubated at 37°C for 2 hours in a water bath. Then, 0.2mL of Schiff reagent was added at room temperature. Thirtyminutes later, the absorbance of the solution was recorded at555 nm in a UV spectrophotometer (Spectronic 20D).

Triplicate samples were run. All the samples were determined with the same procedure. The mucin content was calculated from the standard calibration curve. As comparison,the mucoadhesive potential of EC microspheres was alsoassessed with the above procedure. Each experiment wasperformed 3 times and standard deviation noted.

Adsorption of Mucin on Chitosan Microspheres

Mucin aqueous solution with different concentrations (0.025,0.05,0.1, 0.2, and 0.5 mg/mL) were prepared. Levofloxacin hemihydrate mucoadhesive microspheres(20 mg) weredispersed in the above mucin solutions, vortexed, and shaken at room temperature.[167]Then, thedispersions were centrifuged at 4000 rpm for 2 minutes, and the supernatant wasused for the measurement of the free mucin content. The dataobtained were interpreted usingfollowing Freundlichor Langmuirequations describing the adsorption isotherms:

$$C_{ads} = KC_e^n$$

$$C_{ads} = \frac{aC_e}{b + C_e}$$

Where Cads is the concentration of mucin adsorbed at equilibrium and C_e is the concentration of free mucin at equilibrium.Values of different constants were obtained from the graphsof the above equations. For the Langmuir equation, $1/C_{ads}$was plotted against $1/C_{free}$ to get the constants and for theFreundlich equation, log C_{ads} was plotted against C_{free} to getthe constants.

Compatibility studies

Fourier-Transform Infrared Spectrophotometry (FTIR)

Infrared red spectra for pure Levofloxacin hemihydrate,blank microspheres, Levofloxacin hemihydrate mucoadhesive microspheres were obtained on a FTIR-[Shimadzu (84005)] spectrophotometer using the potassium bromate disk method. 200mg potassium bromate was used for the analysis of 2mg of sample. The scanning range was set into 450–4000 cm^{-1}.

Differential scanning calorimeter (DSC)

The thermal analysis of pure drug, formulations and blank microspheres were carried out using Universal V4.2E TA instruments, to evaluate possible drug-polymer interaction.3mg of sample was

accurately weighed and placed in a 40-µl aluminum pan and sealed with a punched lid. A temperature range of 10–300^{o}C was scanned using a heating rate of 10^{o}C min^{-1}. A nitrogen purge of 50ml min^{-1} was used in the oven.

X-ray powder diffractometry

X-ray powder diffractometry (XRD) study was conducted to study the effect of the formulation process on the crystalline nature of the drug. Powder XRD patterns were recorded on a Bruker AXS D8 Advance diffractometer using Ni-filtered, Cu K[alpha] radiation with 2[theta] interval defined from 20 to 95[degrees] with a step size of 0.05[degrees]. The XRD patterns of pure drug, polymers, formulations and blank microspheres were recorded.

***In vitro* dissolution studies**[168,169]

In vitro drug release from mucoadhesive microspheres was analyzed by using USP dissolution test apparatus 2 (Paddle) with stirrer at 100 rpm (Disso 2000, Labindia). Predetermined quantities of microspheres were placed in bowel. 900 ml of 0.1N HCl (pH 1.2) was used as the dissolution media. Dissolution studies were conducted at 37°C±0.2°C. Samples were taken at suitable time intervals and replaced with the same quantity of fresh dissolution medium. Collected samples filtered through 0.45µm syringeabsorbance was

measured spectrophotometrically (Shimadzu UV/Visible spectrophotometer 2100; Tokyo, Japan) at 293 nm.

Kinetics of drug release

In order to know the drug release mechanism and *in-vitro* drug release kinetics various kinetic models were used. Zero order, first order, Higuchi's,[170] Peppa's[171]modelswere used in this studyand regression coefficient values (R^2) was calculated and analyzed.

Accelerated stability testing according to ICH Q1A (R2)[172]

The optimized formulation (LM6) were stored in a stability chamber (Remi CHM- 10 S®, India) at 40 ± 2°C and humidity of 75 ± 5% RH for 6 months and examined for the drug content, mucoadhesiveness and *in vitro* drug release 0, 30, 90, and 180 days. The zero time samples were used as controls.

Statistical analysis

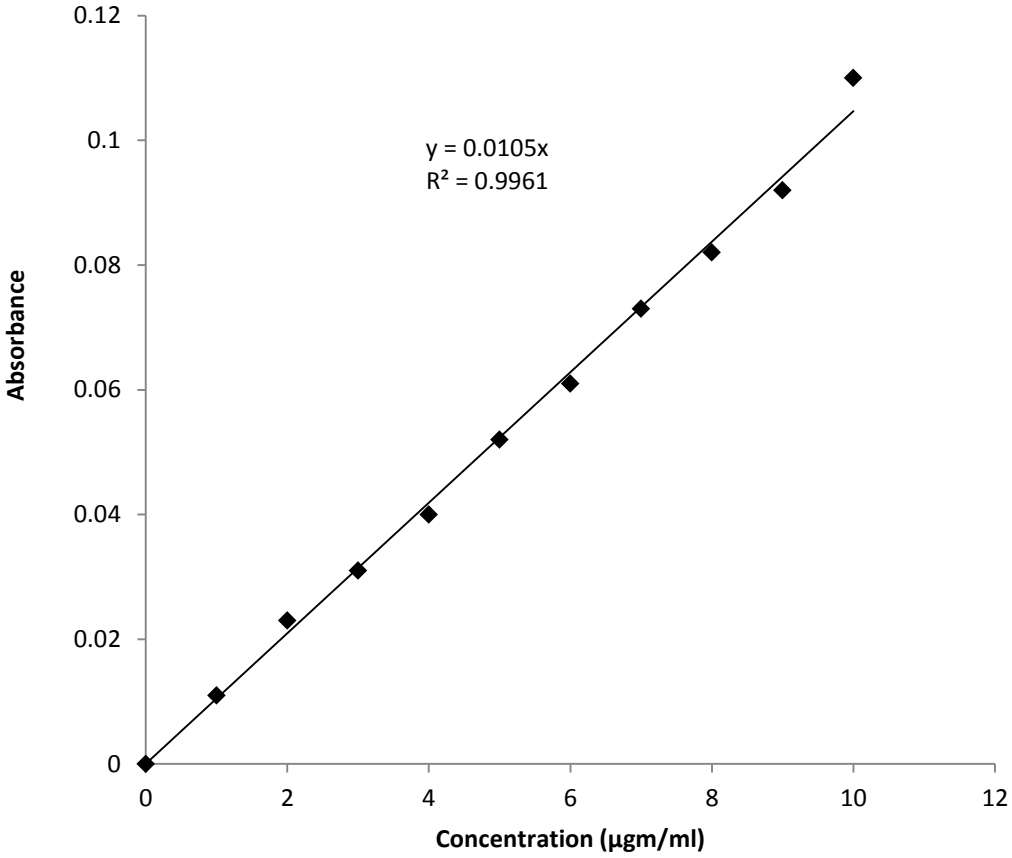
The data obtained from the production yield, encapsulation efficiency, particle size, *in vitro* release studies and *in vivo* studies of microspheres were analyzed statistically by one-way ANOVA using GraphPad Prism software (GraphPad Software) and $P < 0.05$ was considered statistically significant.

8. RESULTS

Table 1: Standard curve of Levofloxacin hemihydrate in 0.1 N HCl

S.No	Concentration (µgm/ml)	Absorbance
1	0	0
2	1	0.011
3	2	0.023
4	3	0.031
5	4	0.040
6	5	0.052
7	6	0.061
8	7	0.073
9	8	0.082
10	9	0.092
11	10	0.110

Figure 1: Standard curve of Levofloxacin hemihydratein 0.1 N HCl



PERCENTAGE YIELD

Table 2: Percentage yield of Levofloxacin hemihydrateloaded mucoadhesive microspheres

S.No	Formulation code	Percentage yield (Mean of three values ± SD)
1	LM1	35.10±1.33
2	LM2	61.37±1.45
3	LM3	81.16±1.35
4	LM4	82.10±1.78
5	LM5	78.29±1.91
6	LM6	82.31±1.48
7	LM7	85.15±1.37
8	LM8	79.52±1.81
9	LM9	89.51±1.88
10	LM10	92.20±1.97

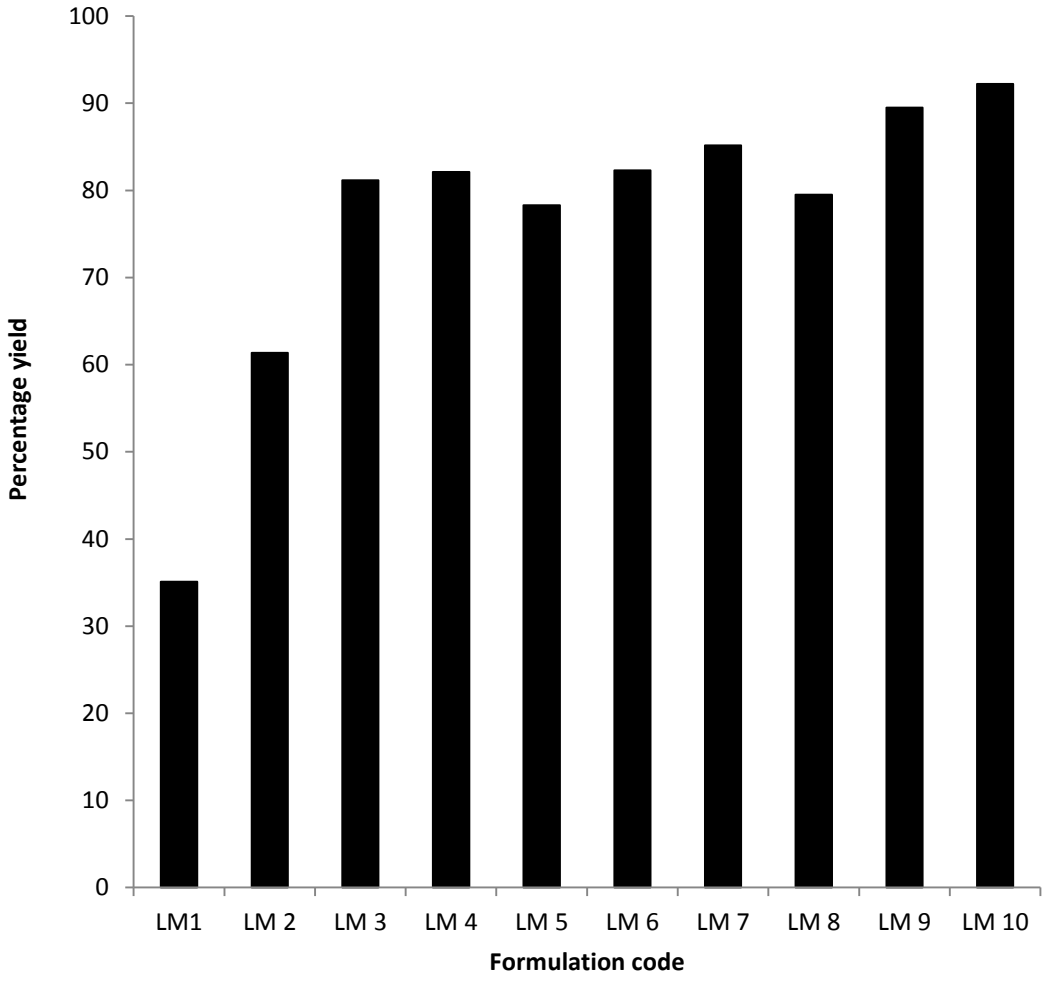


Figure 2: Percentage yield of Levofloxacin hemihydrateloaded mucoadhesive microspheres (Bars represent mean of three values ± SD)

DRUG CONTENT

Table 3: Drug content of levofloxacin hemihydrate loaded mucoadhesive microspheres

S.No	Formulation code	Theoretical drug content (%)	Practical drug content (%) (Mean of three values ± SD)
1	LM1	55.60	29.64±0.42
2	LM 2	45.45	26.56±0.33
3	LM 3	38.46	25.58±0.82
4	LM 4	33.33	27.55±0.32
5	LM 5	40.00	24.56±0.41
6	LM 6	37.04	25.85±0.34
7	LM 7	35.71	25.45±0.65
8	LM 8	40.00	25.95±0.44
9	LM 9	37.04	25.98±0.49
10	LM 10	35.71	25.60±0.61

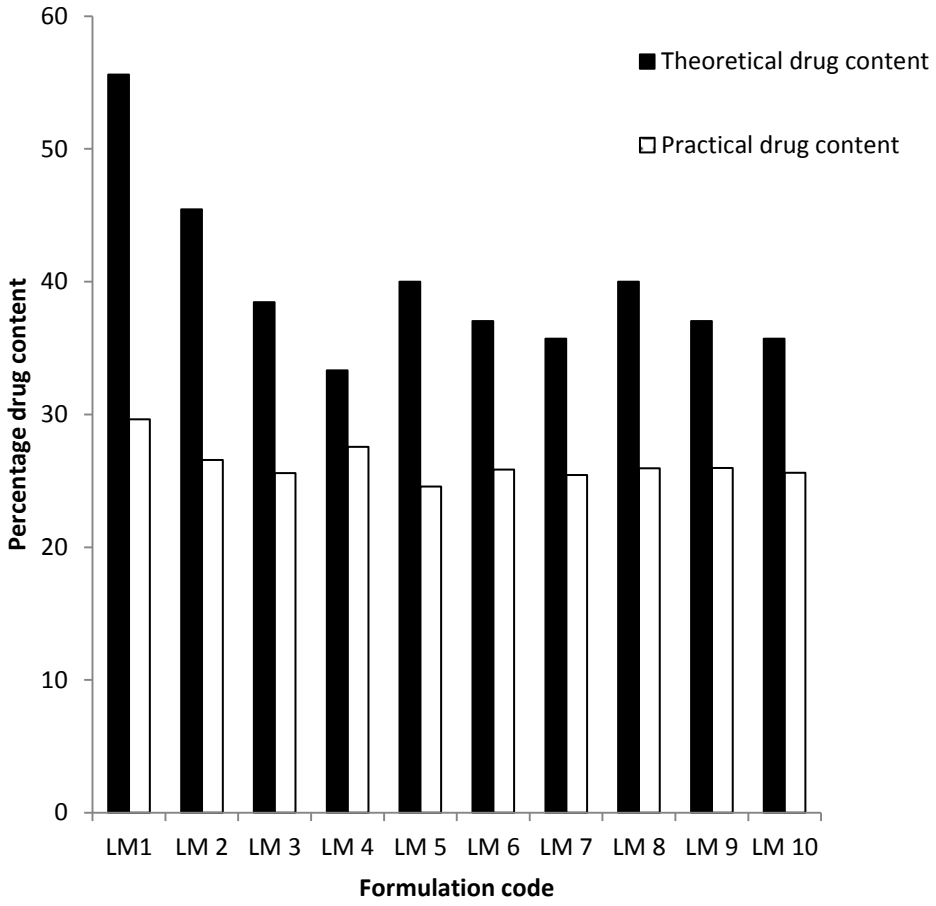


Figure 3: Drug content of Levofloxacin hemihydrateloaded mucoadhesive microspheres (Bars represent mean of three values ± SD)

ENCAPSULATION EFFICIENCY

Table 4: Encapsulation efficiency of Levofloxacin hemihydrate loaded mucoadhesive microspheres

S.No	Formulation code	Percentage drug loaded (Mean of three values ± SD)
1	LM1	52.31±1.16
2	LM 2	60.22±1.92
3	LM 3	69.04±1.55
4	LM 4	86.33±0.67
5	LM 5	65.41±1.14
6	LM 6	72.81±0.19
7	LM 7	74.49±1.72
8	LM 8	67.62±1.14
9	LM 9	73.11±1.51
10	LM 10	75.05±1.81

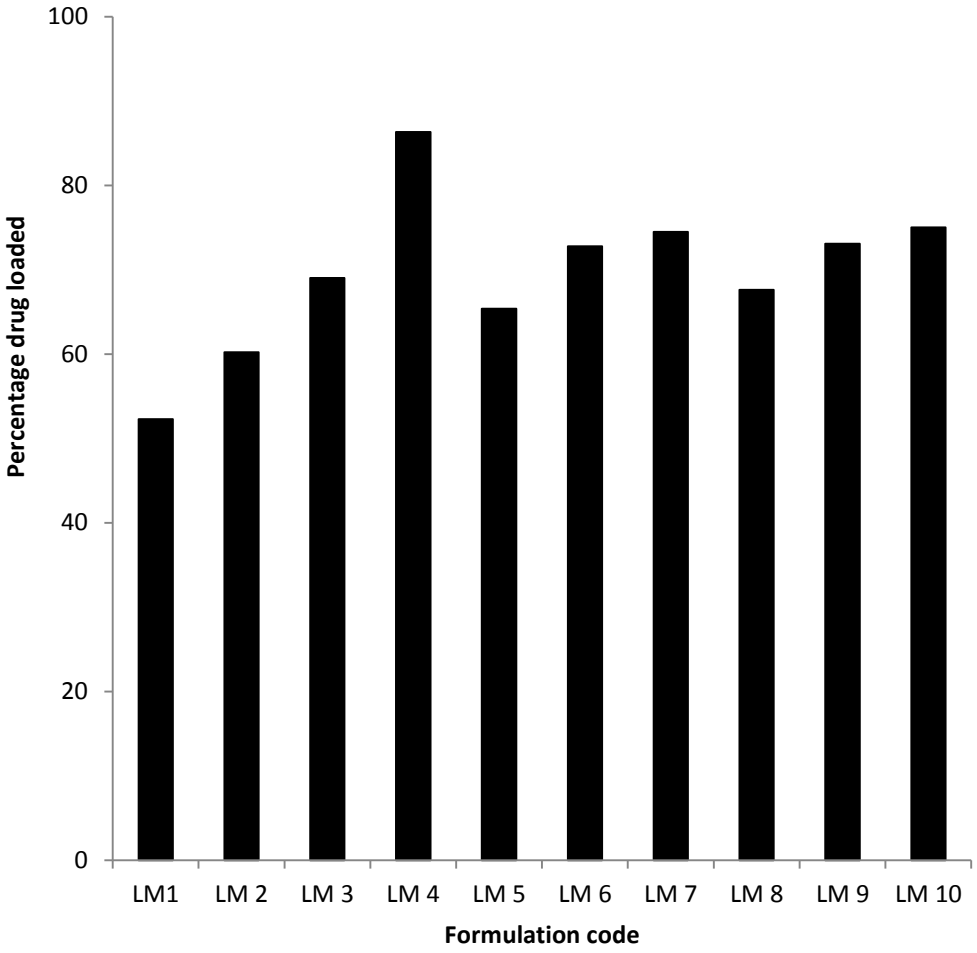


Figure 4: Encapsulation efficiency of Levofloxacin hemihydrate mucoadhesive microspheres (Bars represent mean of three values ± SD)

PARTICLE SIZE DISTRIBUTION

Table 5: Particle size distribution of Levofloxacin hemihydrateloaded mucoadhesive microspheres

S.No	Formulation code	Particle size (µm) (Mean of three values ± SD)
1	LM1	141.17±10.38
2	LM 2	223.61±12.76
3	LM 3	291.57±14.85
4	LM 4	543.91±18.94
5	LM 5	235.59±9.98
6	LM 6	362.48±6.52
7	LM 7	402.40±10.25
8	LM 8	241.37±11.72
9	LM 9	362.23±9.72
10	LM 10	422.11±9.62

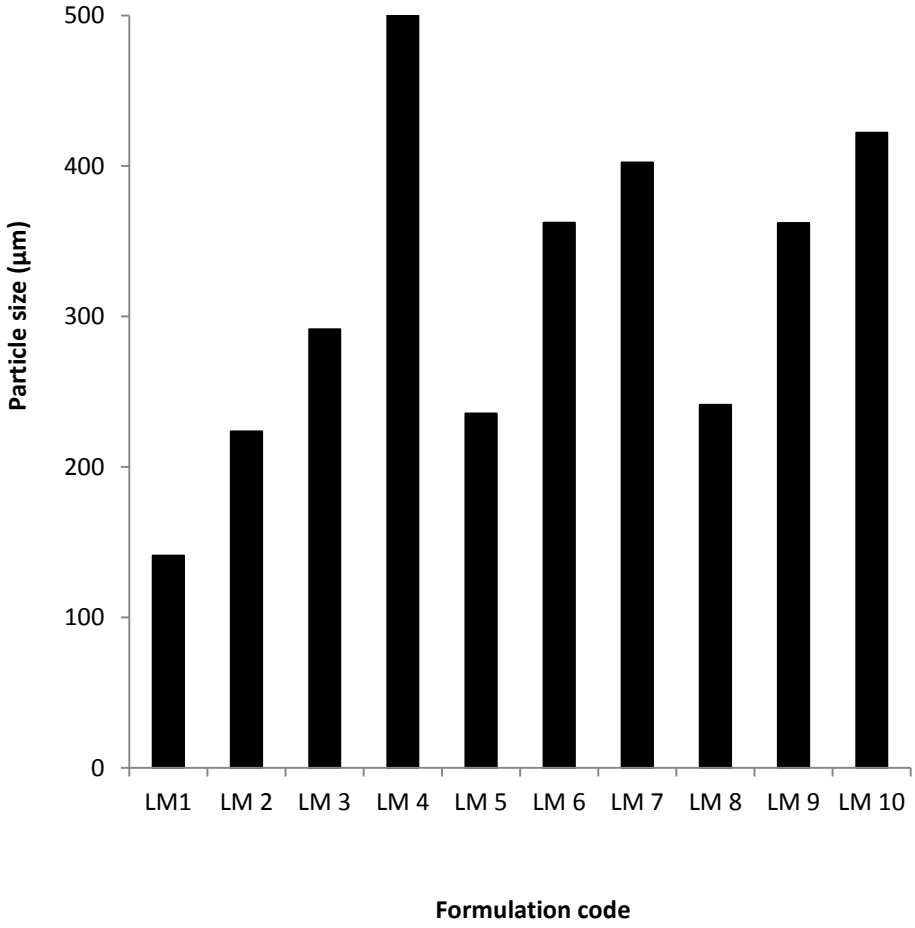


Figure 5: Particle size distribution of Levofloxacin hemihydrate loaded mucoadhesive microspheres (Bars represent mean of three values ± SD)

SCANNING ELECTRON MICROSCOPY (SEM)

SEM photograph of prepared mucoadhesive microspheres of levofloxacin hemihydrate

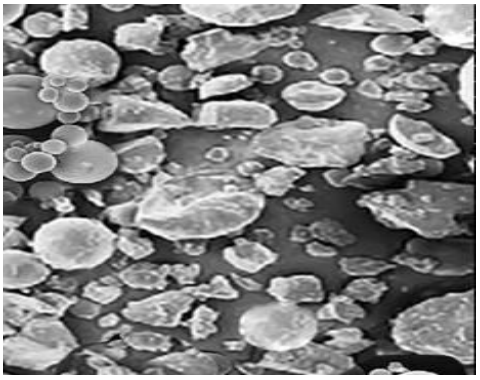


Figure 6: SEM photograph of formulation LM1

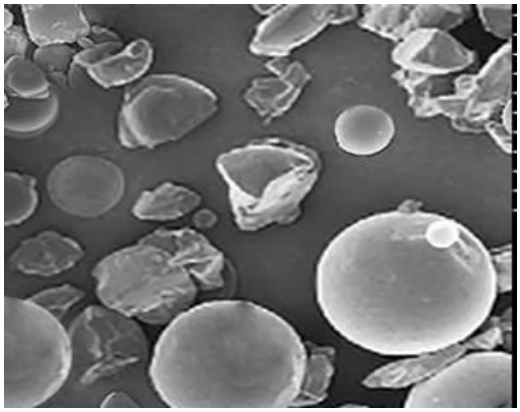


Figure 7: SEM photograph of formulation LM2

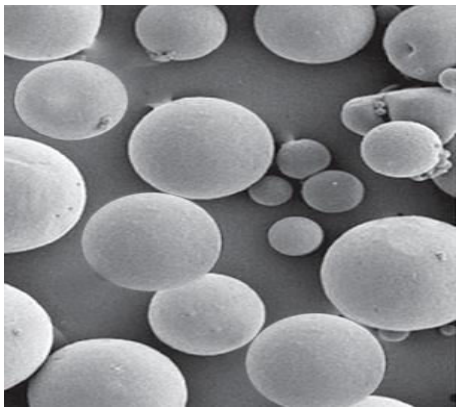


Figure 8: SEM photograph of formulation LM3

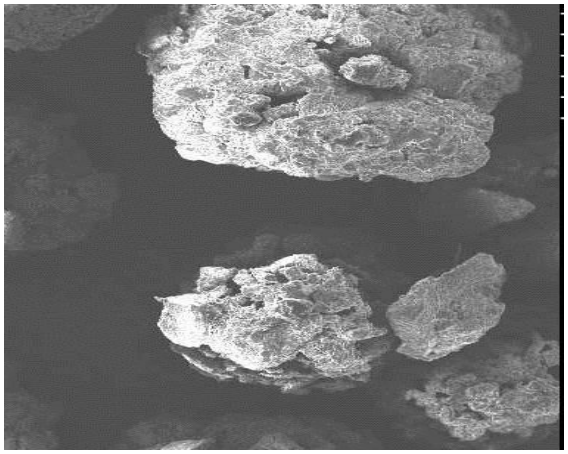


Figure 9: SEM photograph of formulation LM4

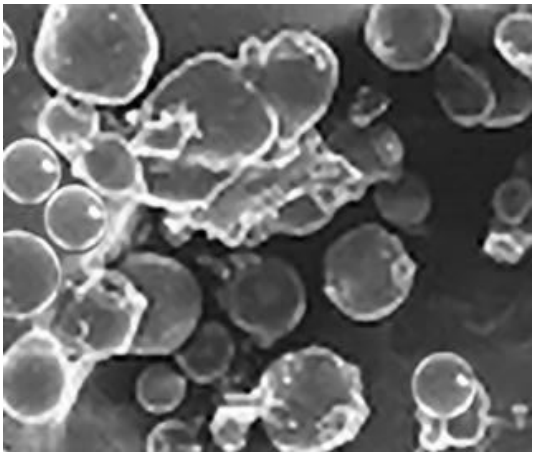


Figure 10: SEM photograph of formulation LM5

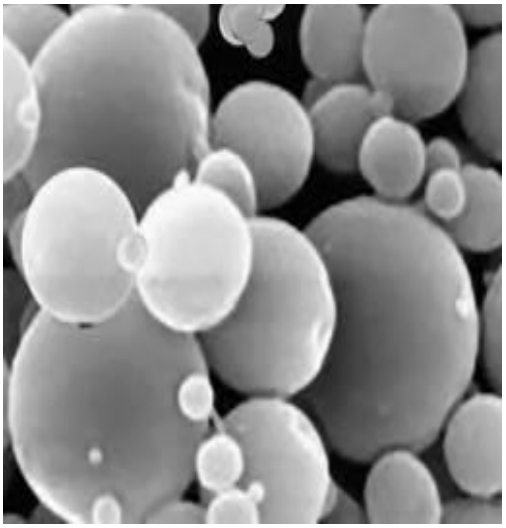


Figure 11: SEM photograph of formulation LM6

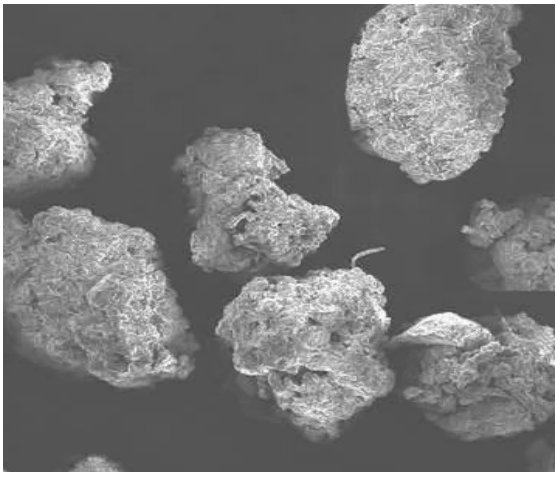


Figure 12: SEM photograph of formulation LM7

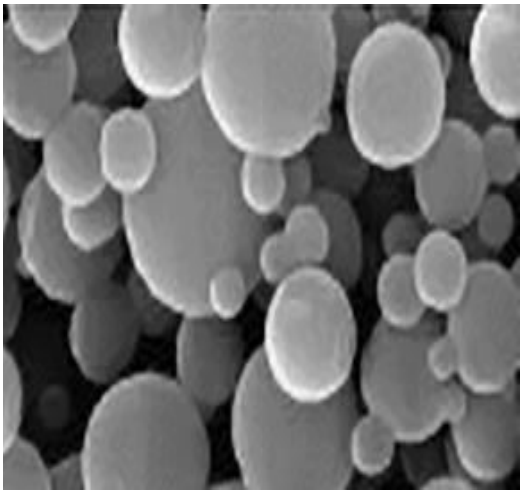


Figure 13: SEM photograph of formulation LM8

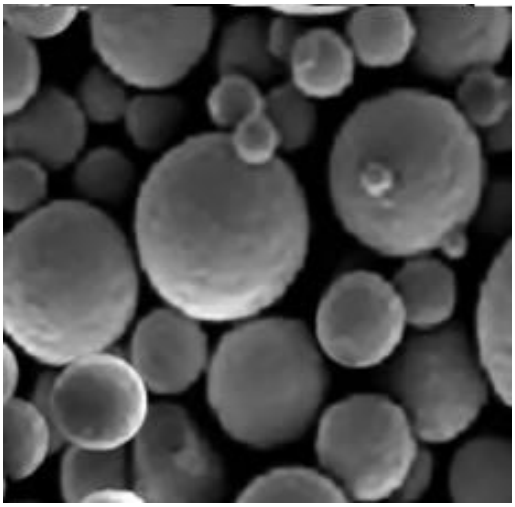


Figure 14: SEM photograph of formulation LM9

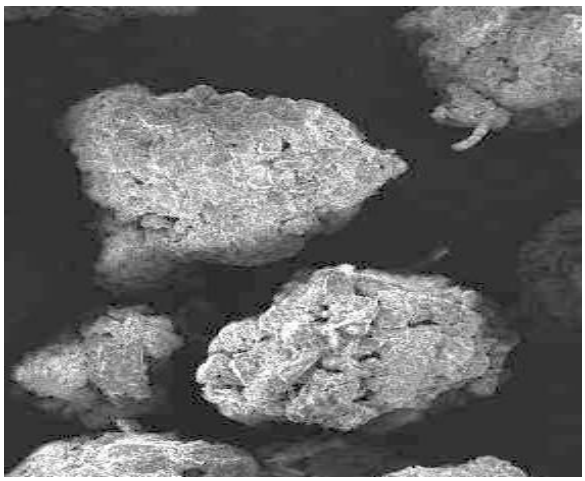


Figure 15: SEM photograph of formulation LM10

MUCOADHESIVENESS EFFICIENCY

Table 6: Mucoadhesiveness efficiency of levofloxacin hemihydrate loaded mucoadhesive microspheres

S.No	Formulation code	Percentage of Mucoadhesiveness (Mean of three values ± SE)
1	LM1	61.11±0.81
2	LM 2	62.09±0.42
3	LM 3	63.33±0.64
4	LM 4	68.57±0.41
5	LM 5	53.55±0.57
6	LM 6	75.47±0.27
7	LM 7	86.54±0.81
8	LM 8	59.35±0.39
9	LM 9	69.20±0.47
10	LM 10	72.10±0.61

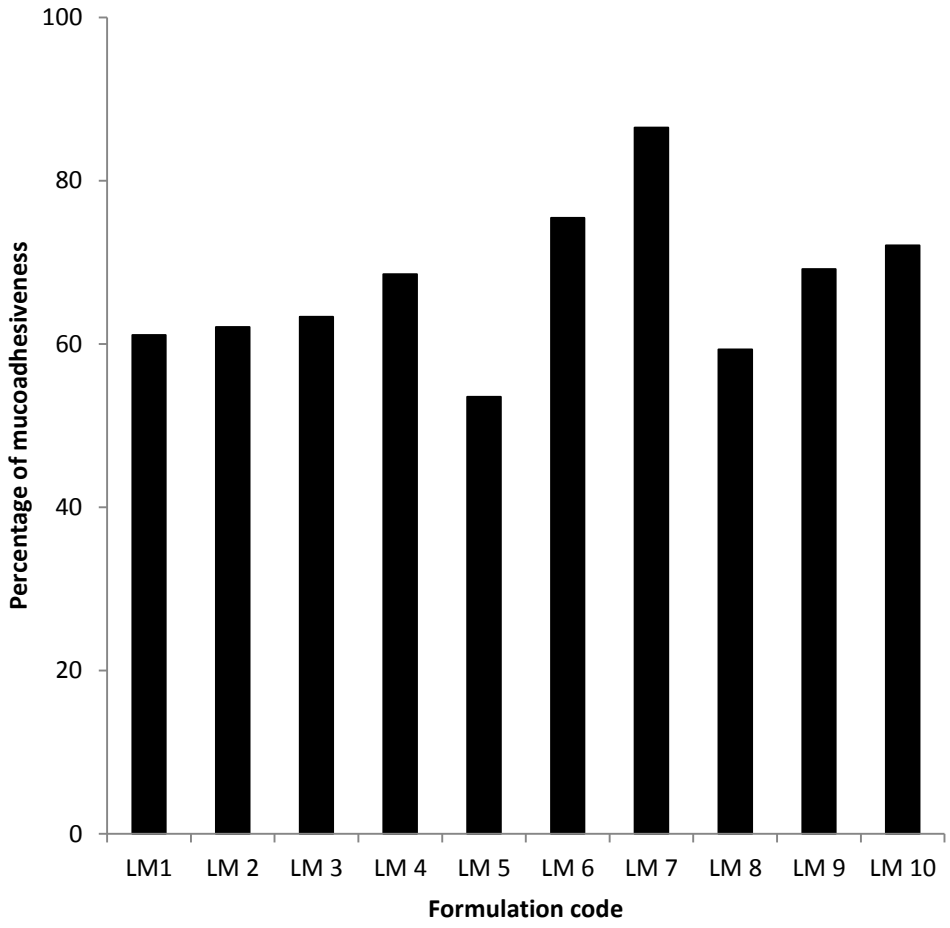


Figure 16: Mucoadhesiveness efficiency of levofloxacin hemihydrate loaded mucoadhesive microspheres (Bars represent mean of three values ± SE)

COMPATIBILITY STUDIES

FOURIER TRANSFORM INFRARED SPECTROPHOTOMETRY (FTIR) STUDIES

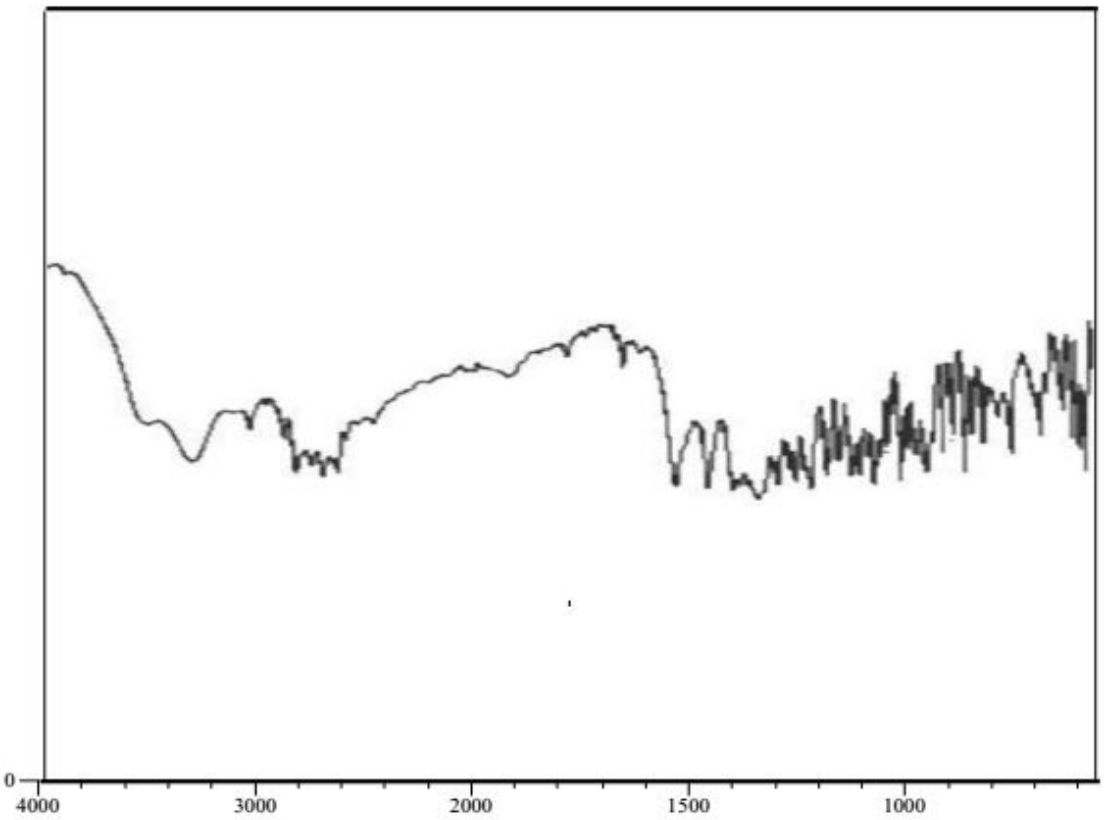


Figure 17: FTIR spectra of Levofloxacin hemihydrate

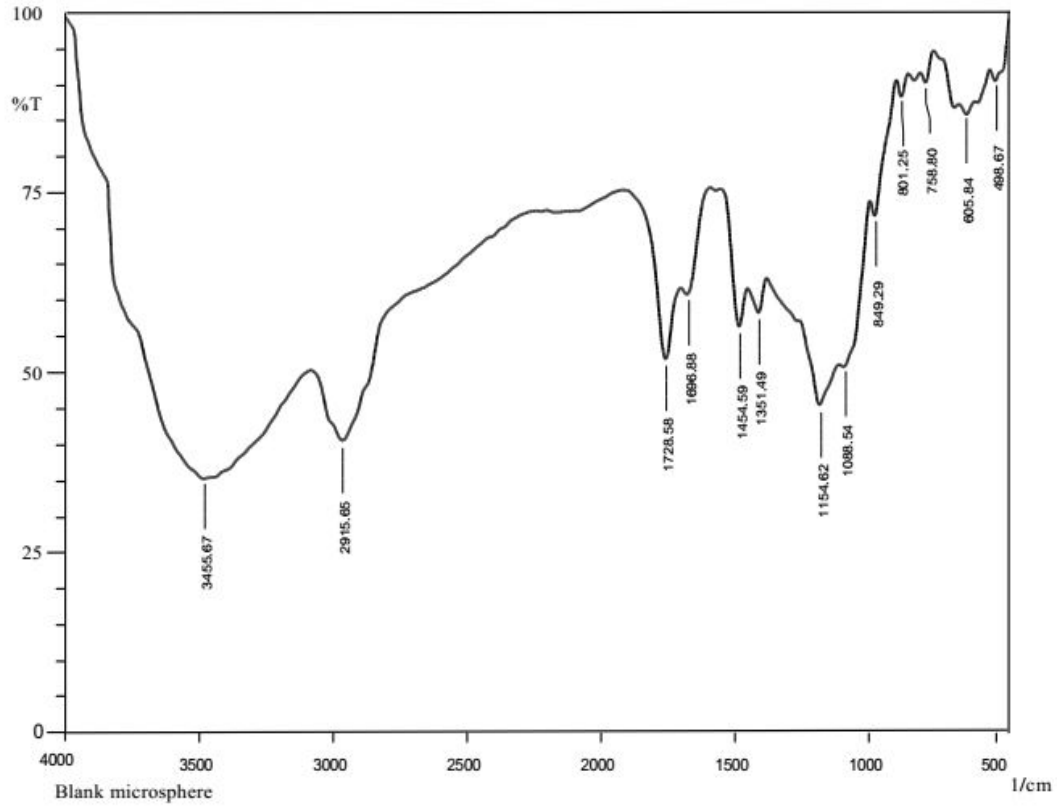


Figure 18:FTIR spectra of Blank mucoadhesive microspheres

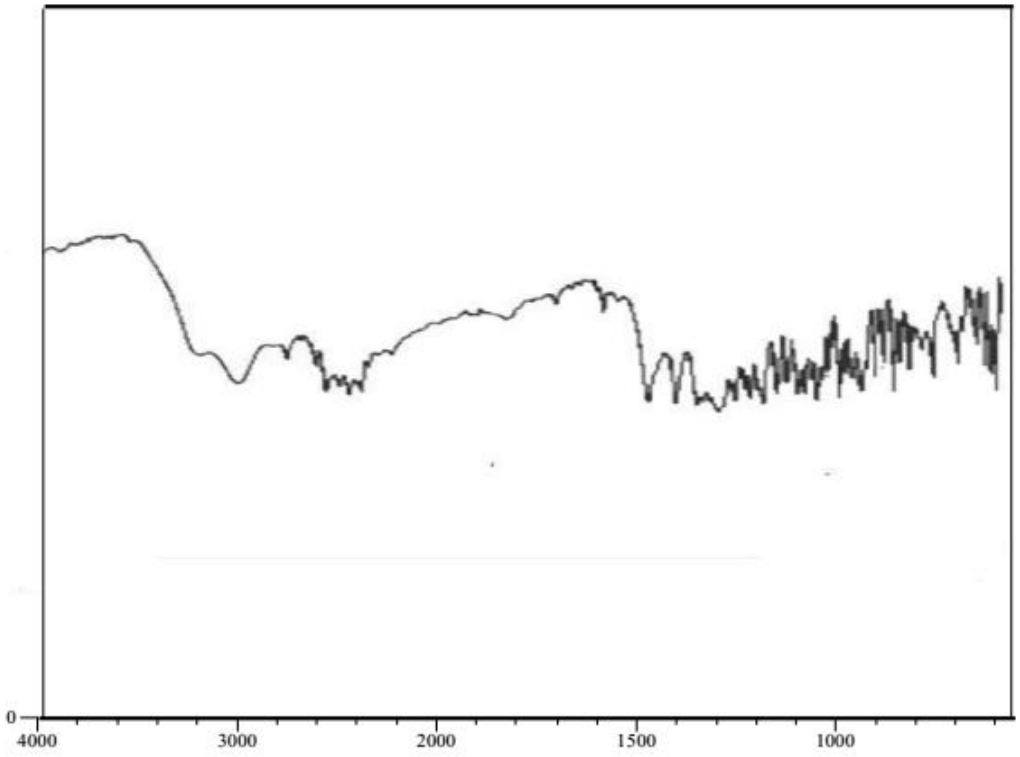


Figure 19: FTIR spectra of Levofloxacin hemihydrateloaded mucoadhesive microspheres

Table 7: Characteristic IR bands of Levofloxacin hemihydratein mucoadhesive microspheres.

Principle peaks	**Levofloxacin hemihydrates (cm^{-1})**	**Levofloxacin hemihydrate loaded microspheres**
–COOH monomeric stretching and bonding	3269 and 1045 cm^{-1}	All the above peaks are present in drug-loaded formulations that confirm the presence of drug in the polymer without any interaction.
alkanes $–CH_3$ and aromatic rings	2846 and 1618 cm^{-1}	
C=O stretching vibration of the COOH group	1721	
C–F	835	

DIFFERENTIAL SCANNING CALORIMETERY (DSC) STUDIES

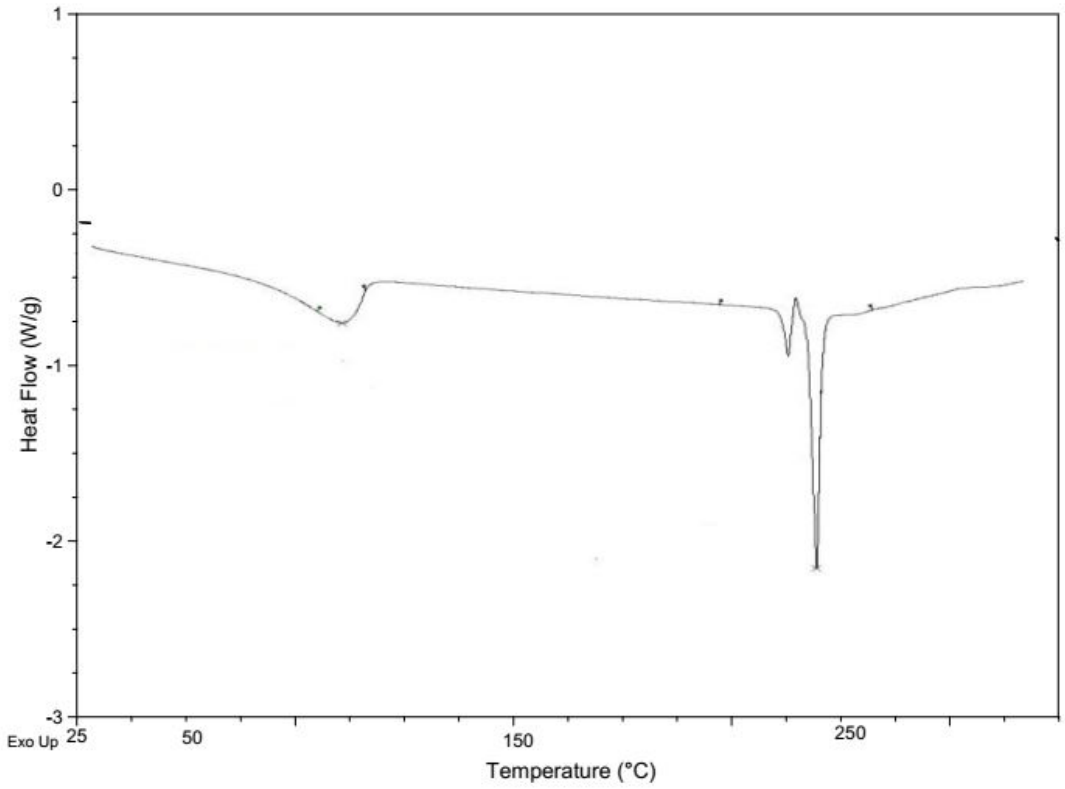


Figure 20: DSC spectra of Levofloxacin hemihydrate

The DSC thermogram of LVFshowed endothermic transitions at 94.2°C and 237.2 °C due to the decomposition of LVF

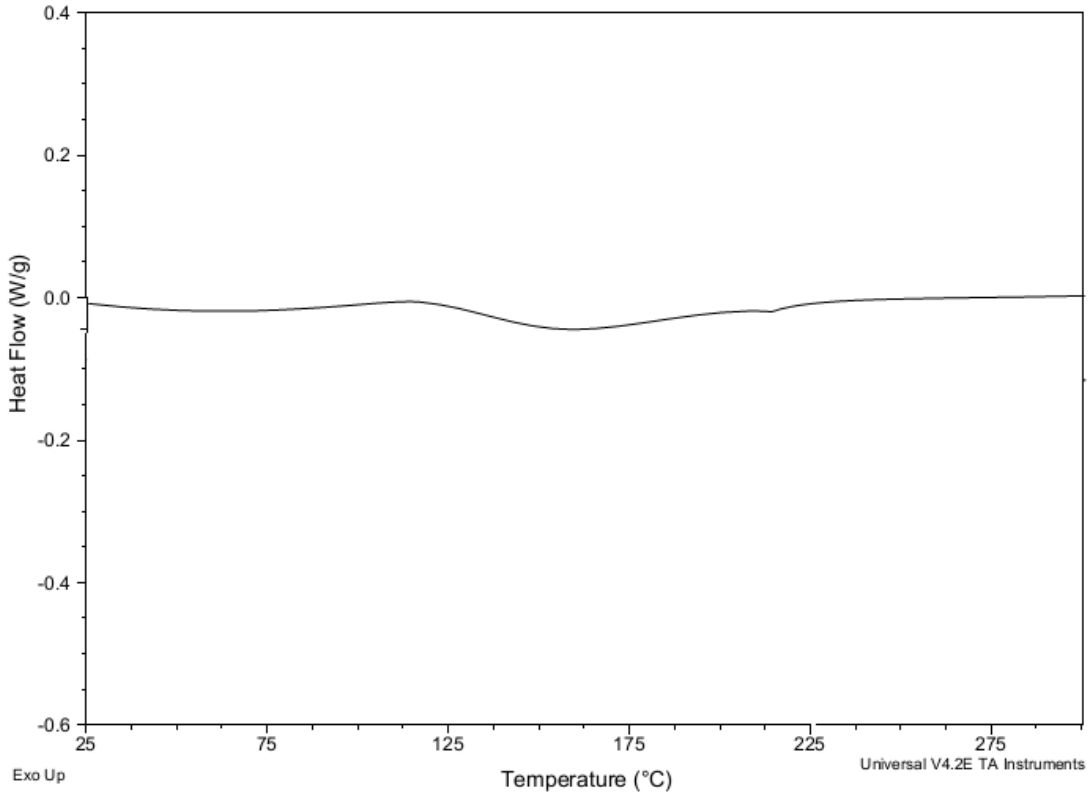


Figure 21: DSC spectra of blank microspheres

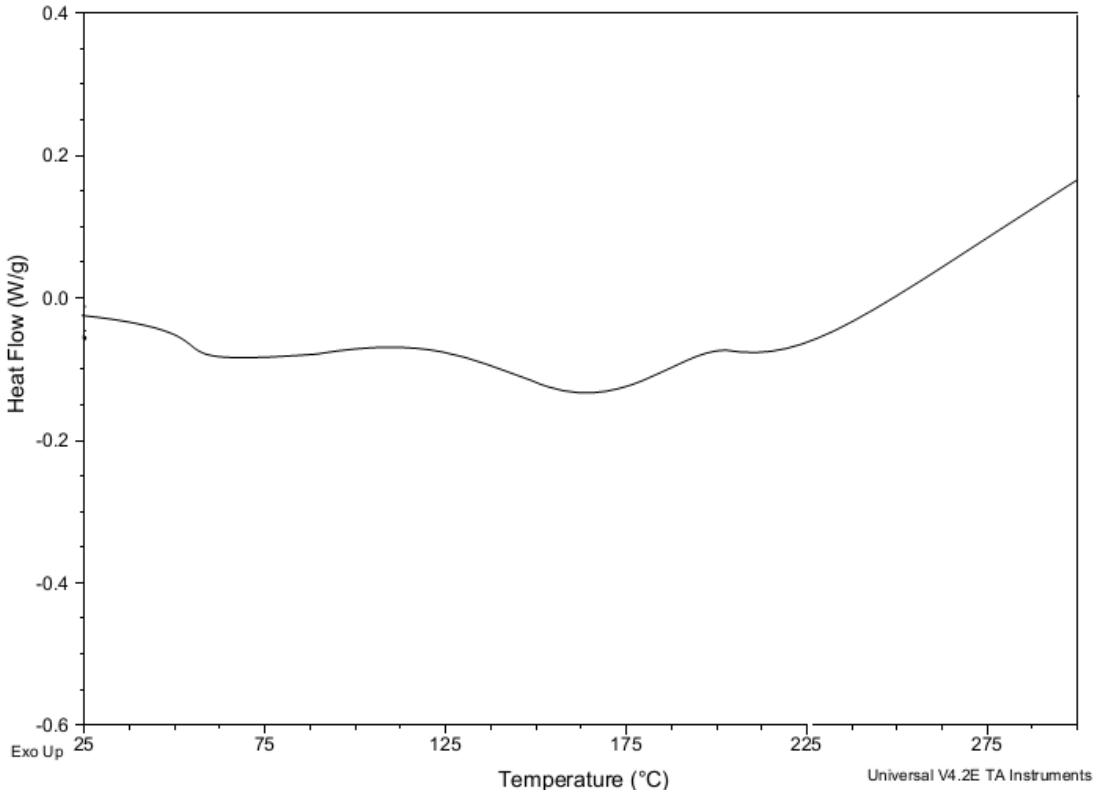


Figure 22: DSC spectra of Levofloxacin hemihydrate loaded mucoadhesive microspheres

Melting peaks of Levofloxacin hemihydrate not observed in the DSC profile of Levofloxacin hemihydrate loaded mucoadhesive microspheres, suggesting a good dispersion of the Levofloxacin hemihydrate in an amorphous state in microspheres.

X-RAY POWDER DIFFRACTOMETRY (XRD) STUDIES

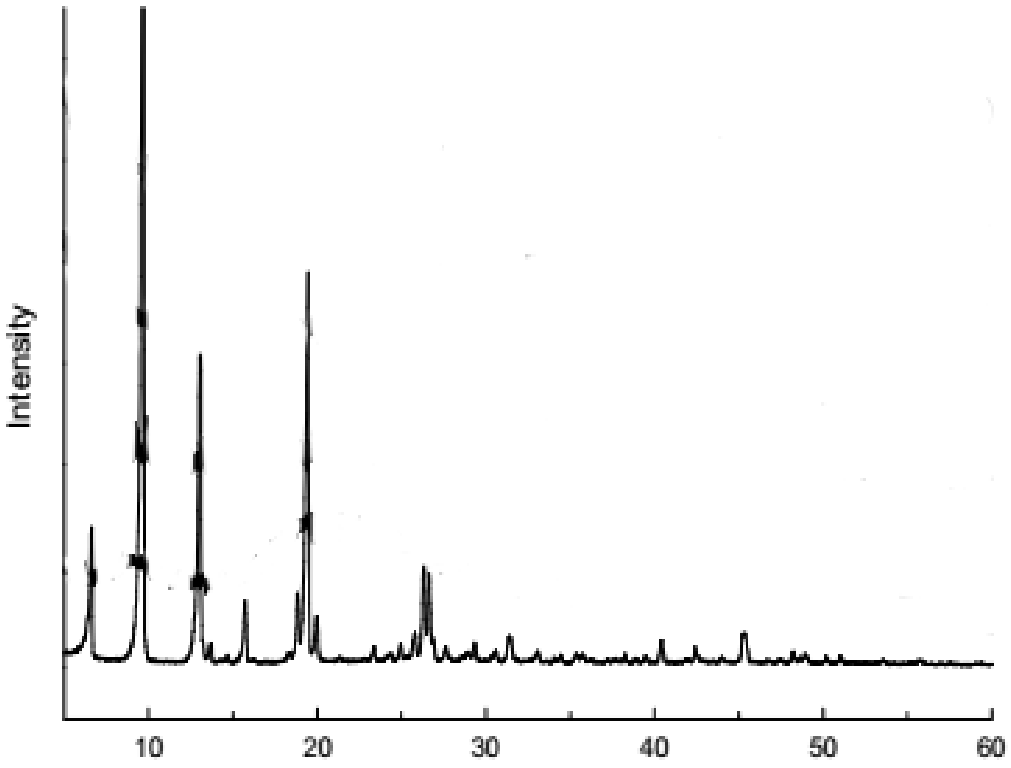


Figure 23: XRD spectra of Levofloxacin hemihydrate

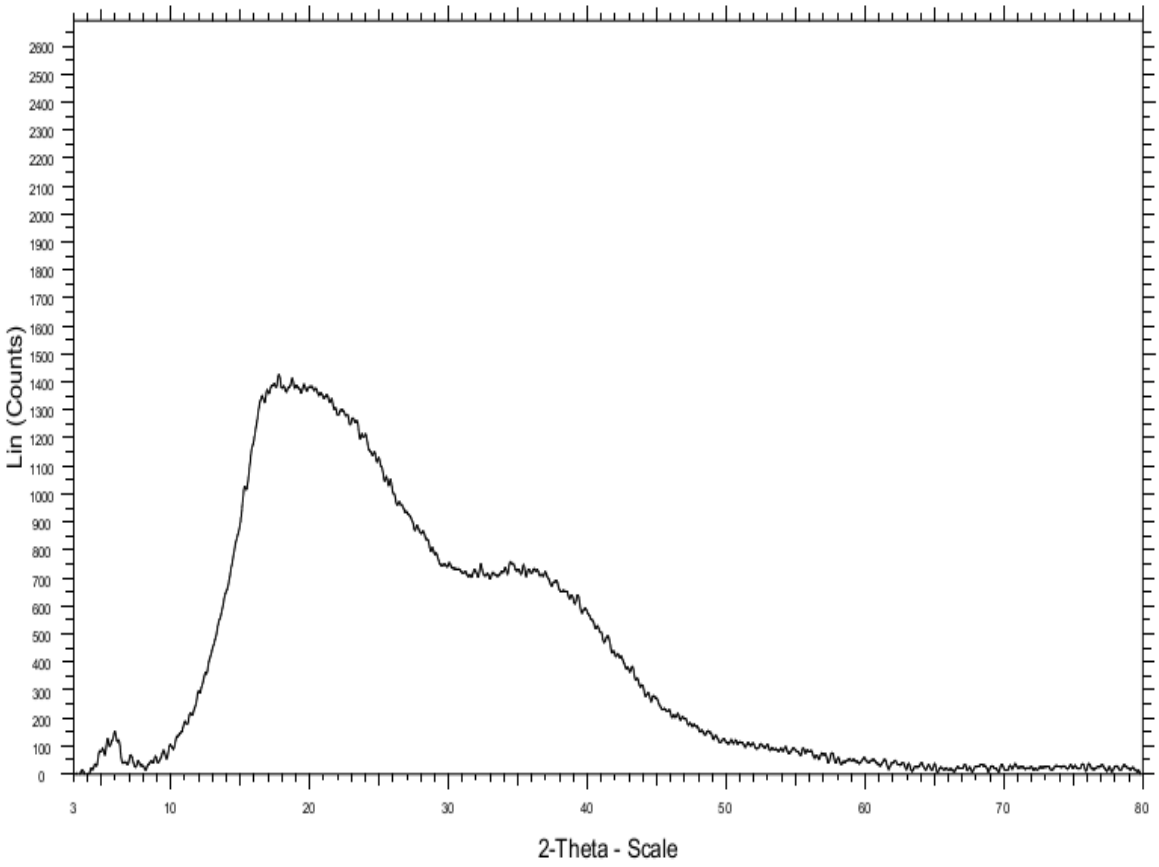


Figure 24: XRD spectra of carbopol 974P

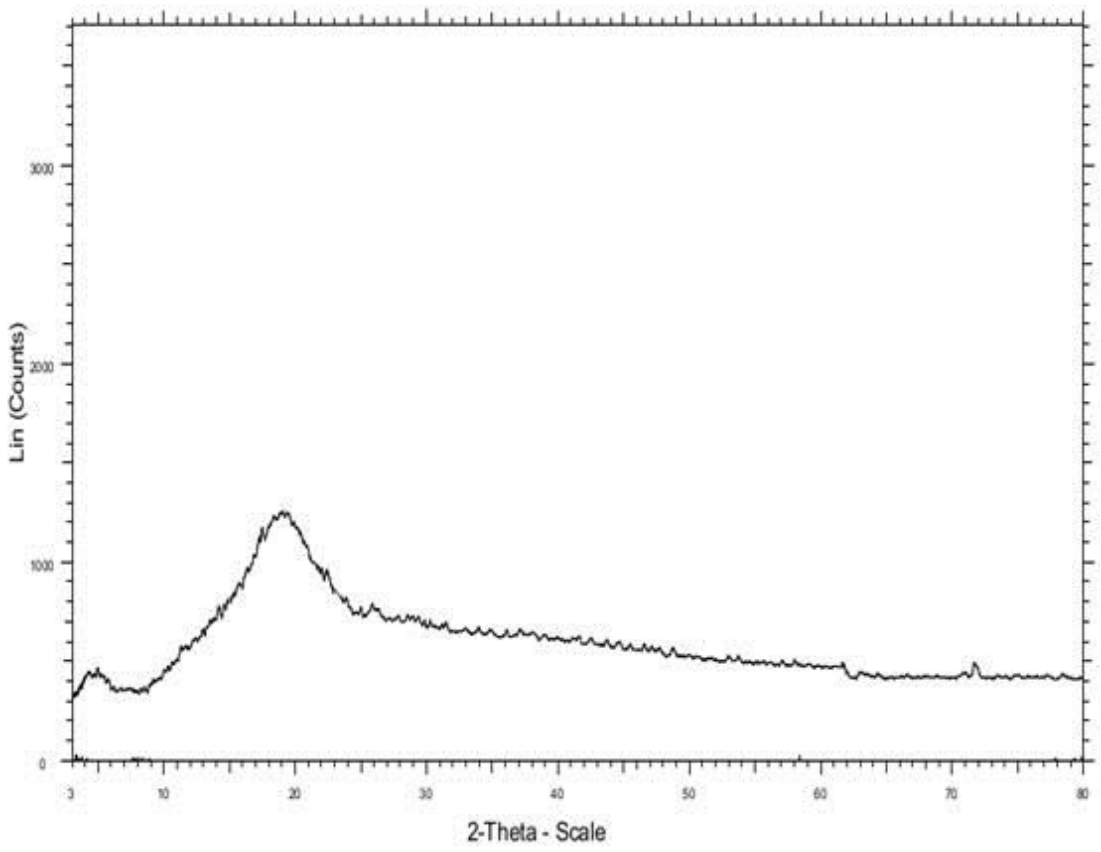


Figure 25: XRD spectraof HPMC K4M

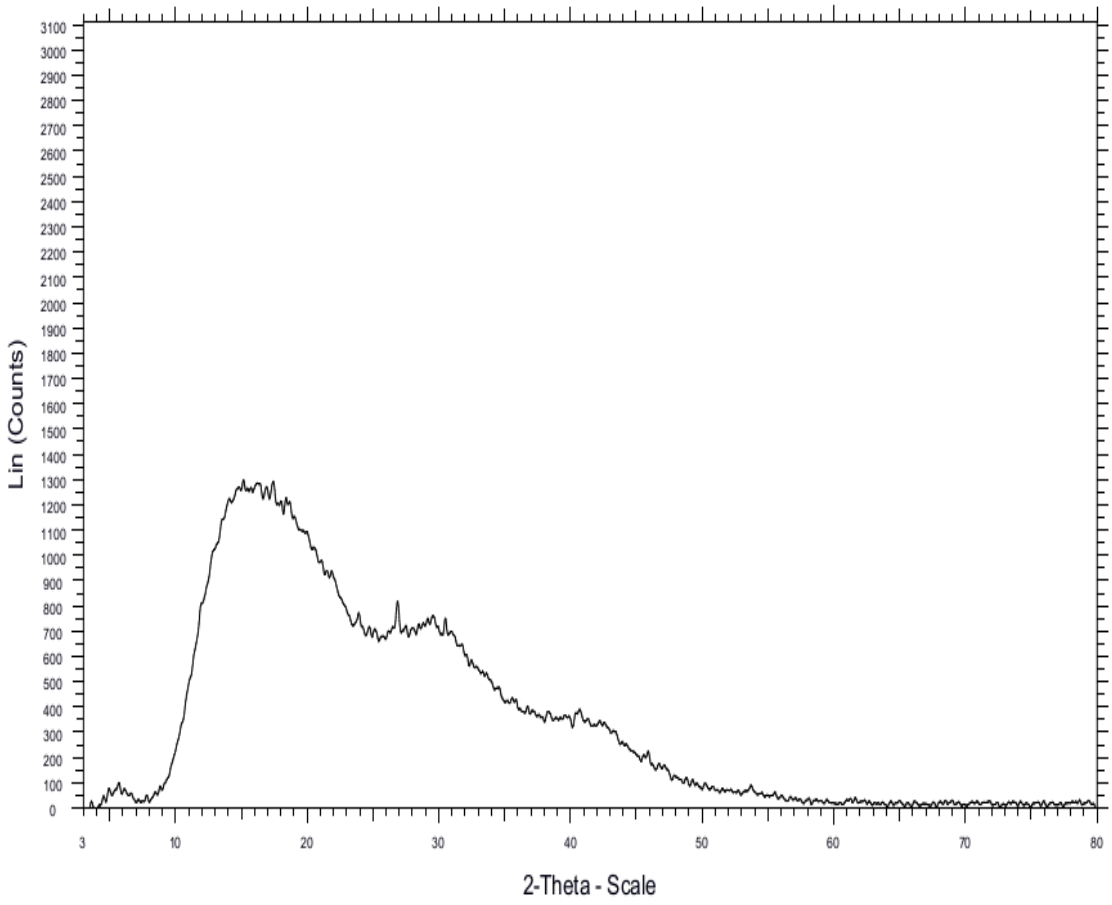


Figure 26: XRD spectra of Eudragit RS 100

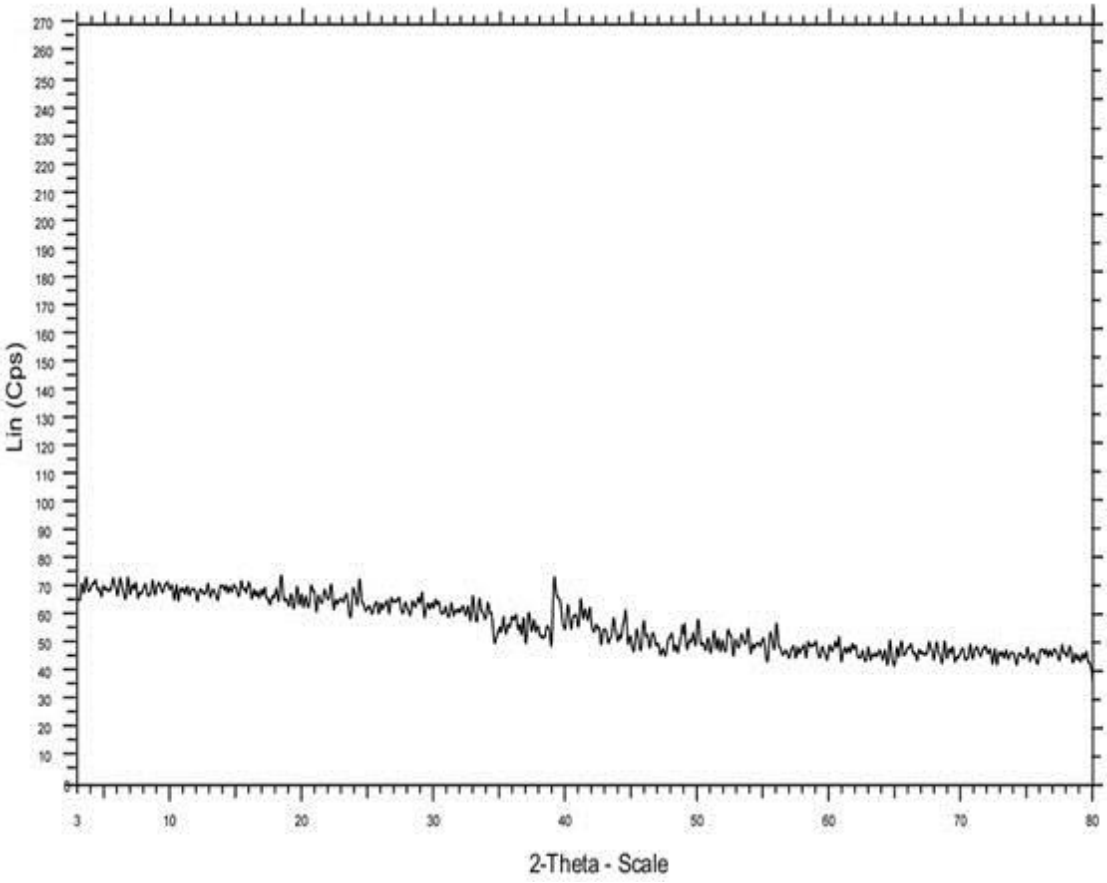


Figure 27: XRD spectra of Levofloxacin hemihydrate loaded mucoadhesive microspheres

No peaks associated with the Levofloxacin hemihydrate were observed in the XRD patterns Levofloxacin hemihydrate loaded mucoadhesive microspheres, suggesting that the antibiotic couldbe molecularly dispersed within the matrix in an amorphousstate.

IN VITRO DISSOLUTION STUDIES

Table 8: *In vitro* release profile of Levofloxacin hemihydrate loaded mucoadhesive microspheres (Formulation LM1)

Time (hours)	Cumulative Percentage drug Released*	Standard Deviation**
0	0	0
1	52.08	1.16
2	80.33	1.21
3	99.62	0.34
4	-	-
5	-	-
6	-	-
7	-	-

*Each reading is an average of three determinations
**Standard deviation of three determinations

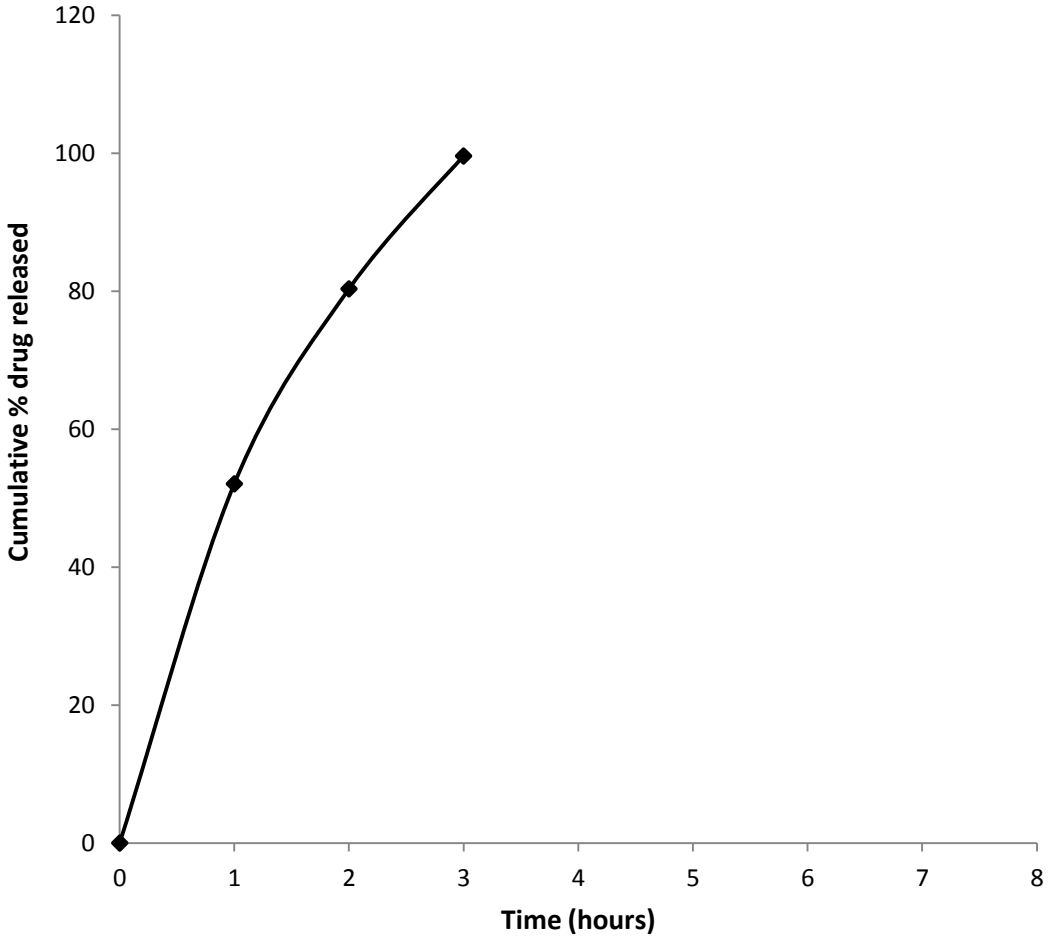


Figure 28: *In vitro* release profile of Levofloxacin hemihydrate loaded mucoadhesive microspheres (Formulation LM1)

(Bars represent mean of three values ± SD)

Table 9: *In vitro* release profile of Levofloxacin hemihydrate loaded mucoadhesive microspheres (Formulation LM2)

Time (hours)	Cumulative Percentage drug Released*	Standard Deviation**
0	0	0
1	45.61	1.15
2	64.07	2.06
3	81.29	1.32
4	99.25	0.19
5	-	-
6	-	-
7	-	-

*Each reading is an average of three determinations
**Standard deviation of three determinations

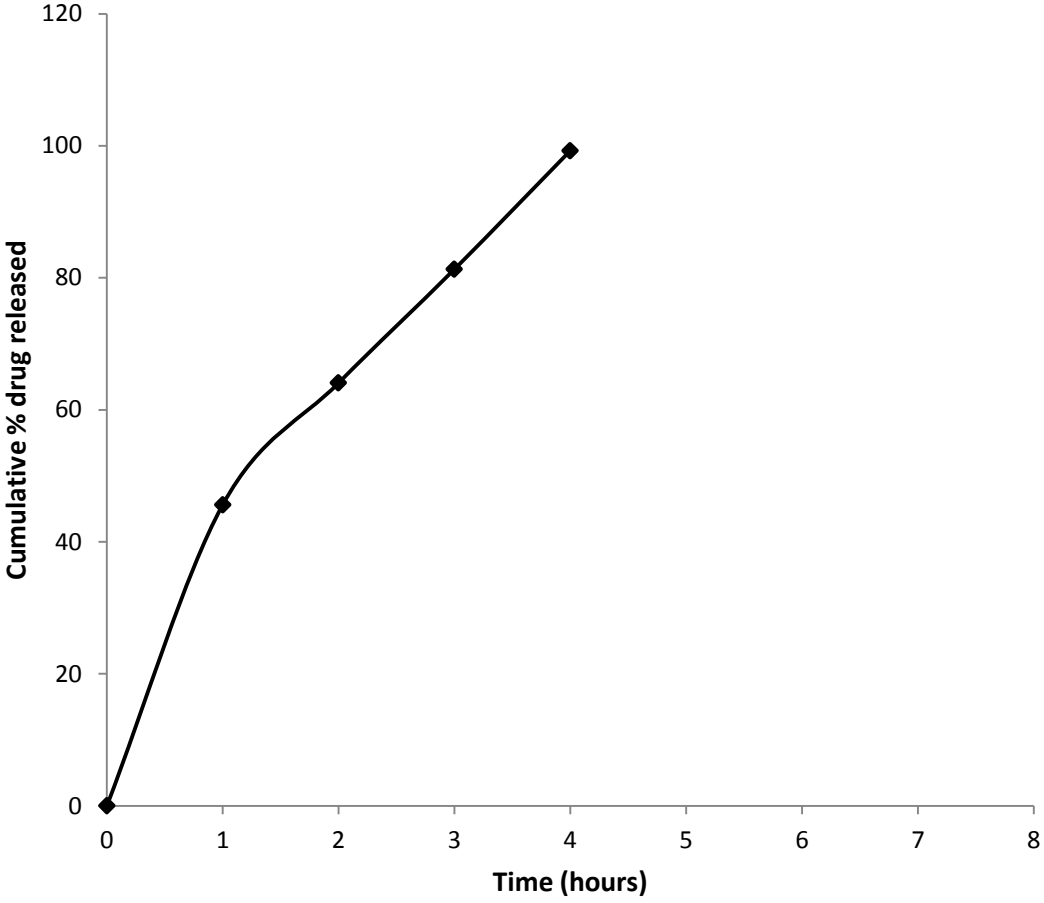


Figure 29: *In vitro* release profile of Levofloxacin hemihydrate loaded mucoadhesive microspheres (Formulation LM2)

(Bars represent mean of three values ± SD)

Table 10: *In vitro* release profile of Levofloxacin hemihydrate loaded mucoadhesive microspheres (Formulation LM3)

Time (hours)	Cumulative Percentage drug Released*	Standard Deviation**
0	0	0
1	32.15	2.31
2	52.16	2.18
3	66.67	1.34
4	82.03	1.84
5	99.73	0.14
6	-	-
7	-	-

*Each reading is an average of three determinations
**Standard deviation of three determinations

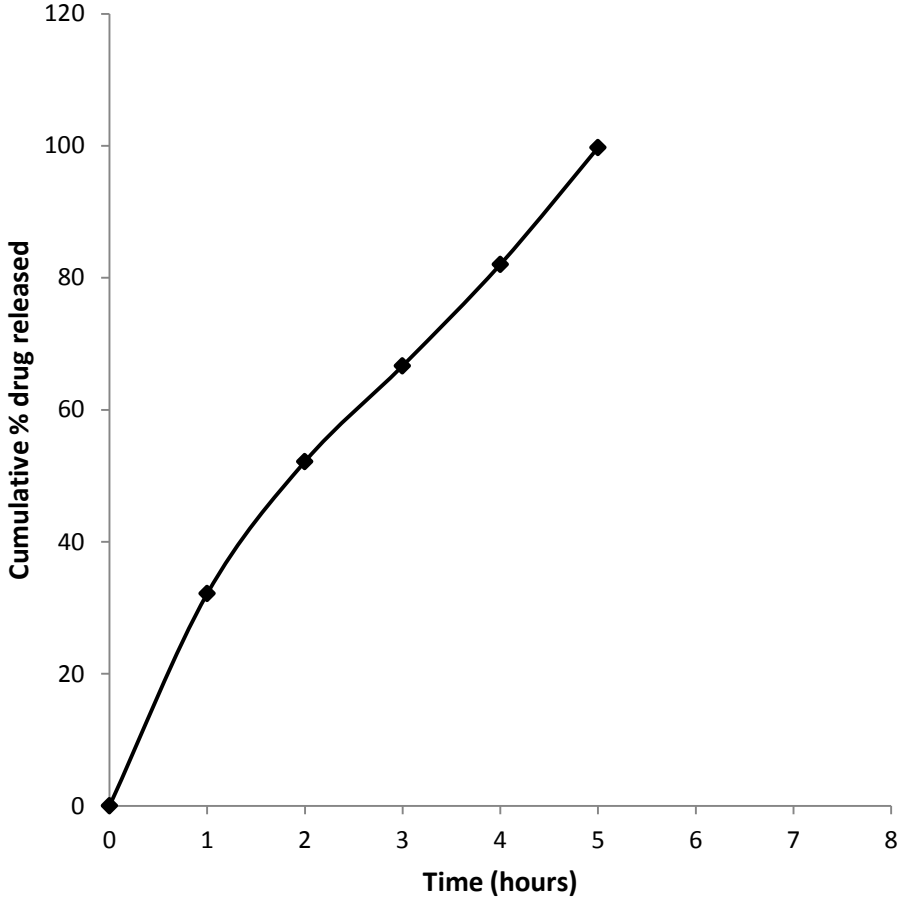


Figure 30: *In vitro* release profile of Levofloxacin hemihydrate loaded mucoadhesive microspheres (Formulation LM3)

(Bars represent mean of three values ± SD)

Table 11: *In vitro* release profile of amLevofloxacin hemihydrate oxicillin trihydrate loaded mucoadhesive microspheres (Formulation LM4)

Time (hours)	Cumulative Percentage drug Released*	Standard Deviation**
0	0	0
1	23.12	1.58
2	40.52	1.96
3	51.62	1.70
4	63.59	2.18
5	74.58	1.39
6	85.61	1.75
7	96.07	1.02

*Each reading is an average of three determinations
**Standard deviation of three determinations

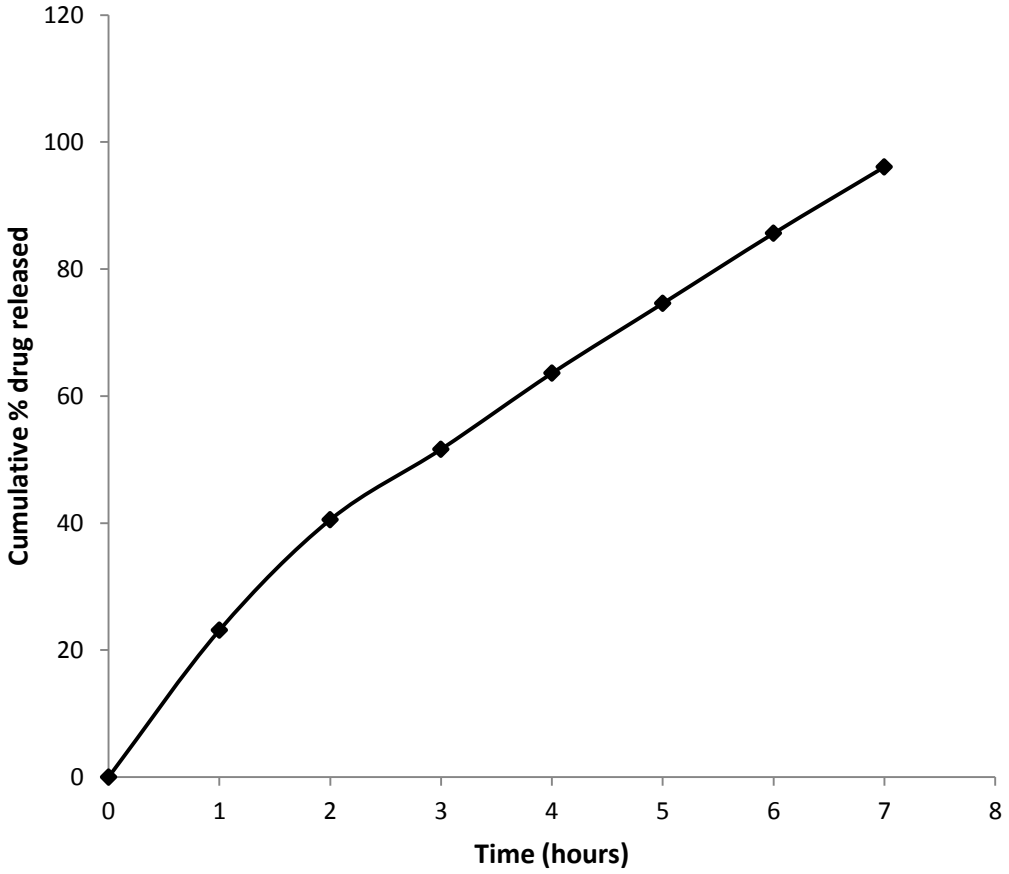


Figure 31: *In vitro* release profile of Levofloxacin hemihydrate loaded mucoadhesive microspheres (Formulation LM4)

(Bars represent mean of three values ± SD)

Table 12: *In vitro* release profile of Levofloxacin hemihydrateloaded mucoadhesive microspheres (Formulation LM5)

Time (hours)	Cumulative Percentage drug Released*	Standard Deviation**
0	0	0
1	51.13	1.69
2	67.28	1.78
3	83.41	2.37
4	99.78	0.08
5	-	-
6	-	-
7	-	-

*Each reading is an average of three determinations
**Standard deviation of three determinations

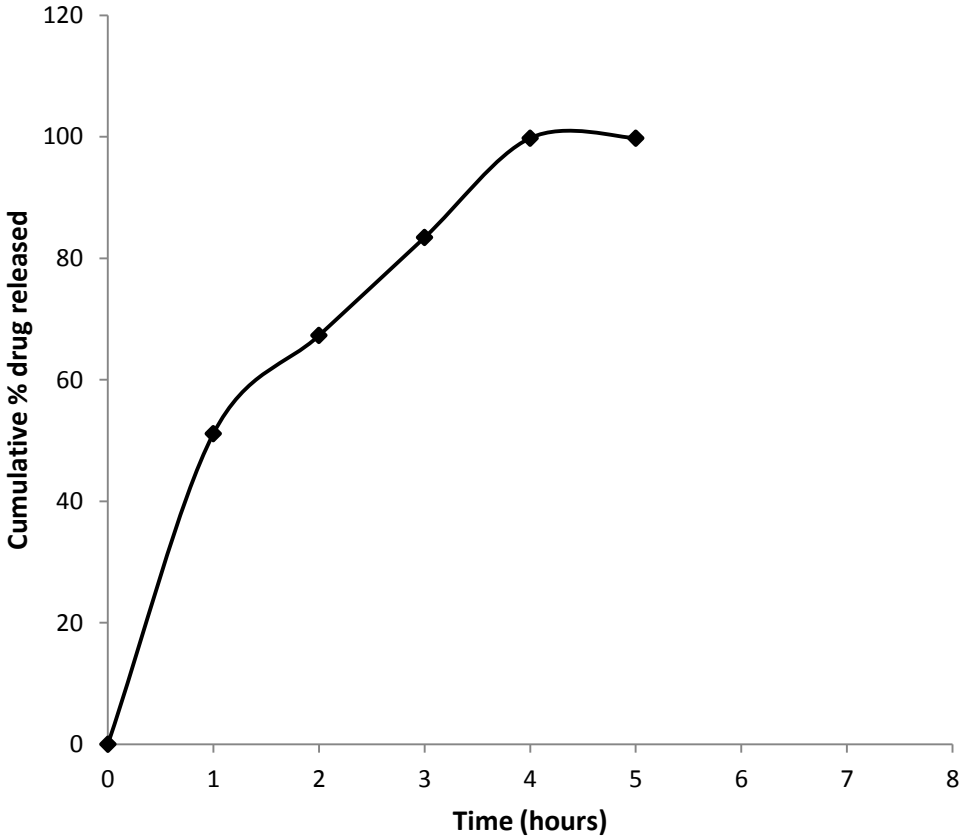


Figure 32: *In vitro* release profile of Levofloxacin hemihydrate loaded mucoadhesive microspheres (Formulation LM5)

(Bars represent mean of three values ± SD)

Table 13: *In vitro* release profile of Levofloxacin hemihydrate loaded mucoadhesive microspheres (Formulation LM6)

Time (hours)	Cumulative Percentage drug Released*	Standard Deviation**
0	0	0
1	30.01	1.61
2	45.63	1.22
3	63.67	1.09
4	71.81	2.61
5	84.22	2.05
6	99.62	0.23
7	-	-

*Each reading is an average of three determinations
**Standard deviation of three determinations

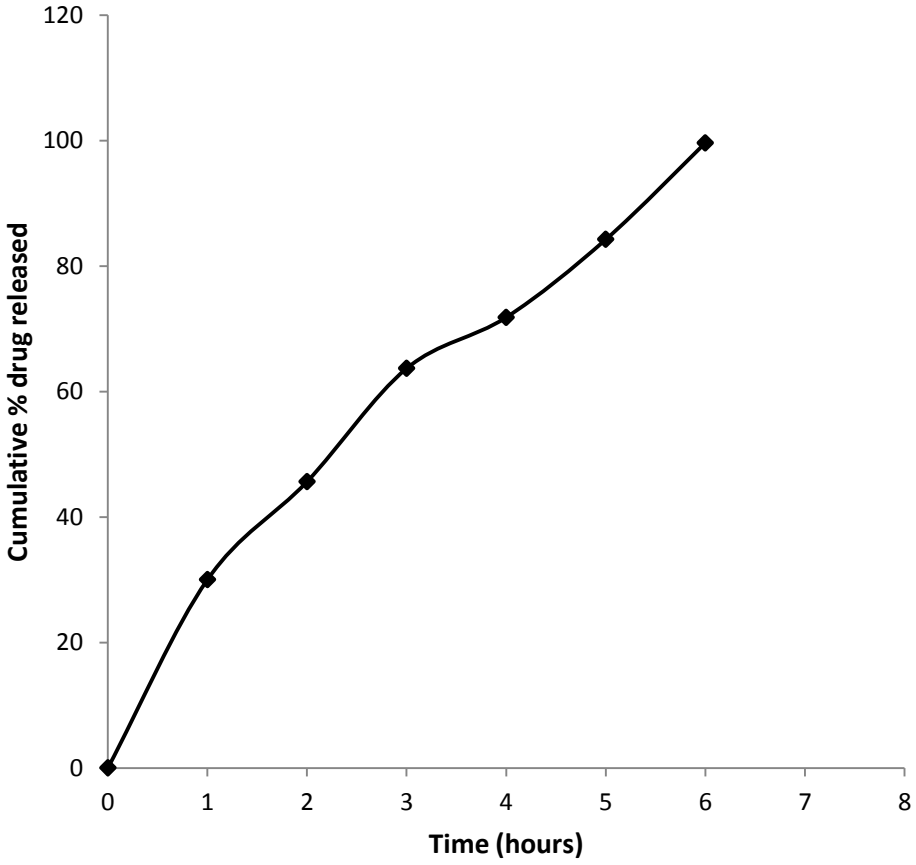


Figure 33: *In vitro* release profile of Levofloxacin hemihydrateloaded mucoadhesive microspheres (Formulation LM6) (Bars represent mean of three values ± SD)

Table 14: *In vitro* release profile of Levofloxacin hemihydrateloaded mucoadhesive microspheres (Formulation LM7)

Time (hours)	Cumulative Percentage drug Released*	Standard Deviation**
0	0	0
1	26.51	1.25
2	33.73	1.22
3	52.25	2.68
4	63.84	2.78
5	74.08	2.27
6	85.15	1.65
7	99.68	0.28

*Each reading is an average of three determinations
**Standard deviation of three determinations

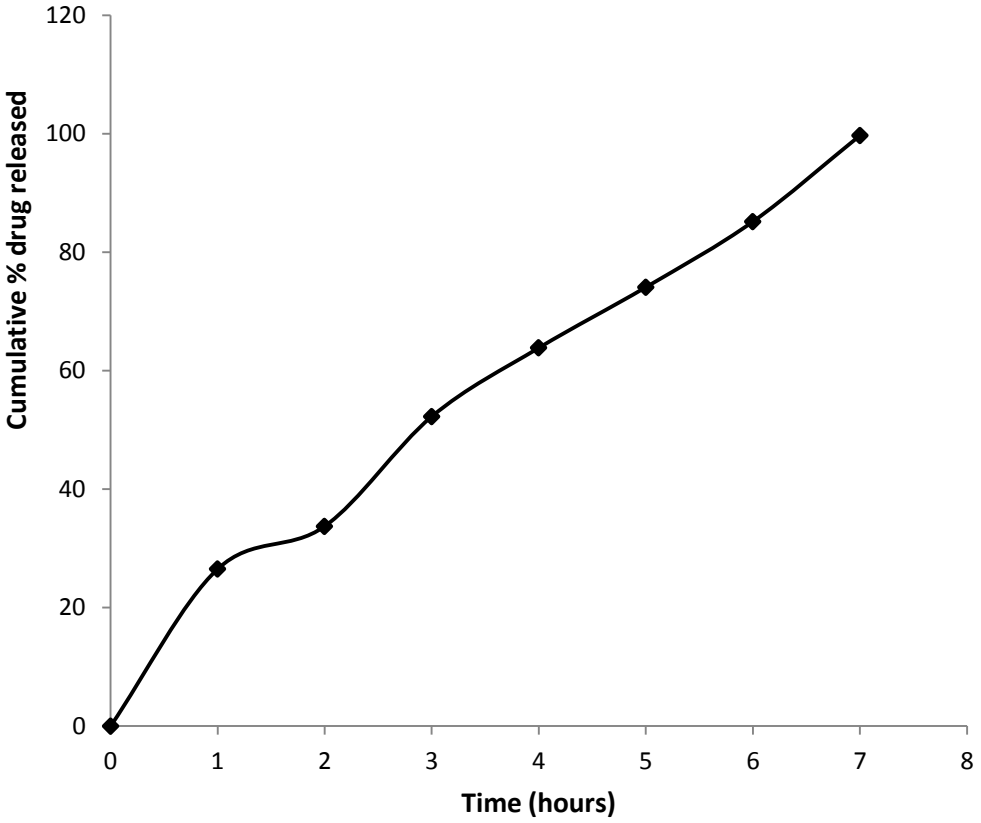


Figure 34: *In vitro* release profile of Levofloxacin hemihydrate loaded mucoadhesive microspheres (Formulation LM7) (Bars represent mean of three values ± SD)

Table 15: *In vitro* release profile of Levofloxacin hemihydrate loaded mucoadhesive microspheres (Formulation LM8)

Time (hours)	Cumulative Percentage drug Released*	Standard Deviation**
0	0	0
1	38.18	0.91
2	61.38	1.50
3	79.31	1.12
4	99.69	0.27
5	-	-
6	-	-
7	-	-

*Each reading is an average of three determinations
**Standard deviation of three determinations

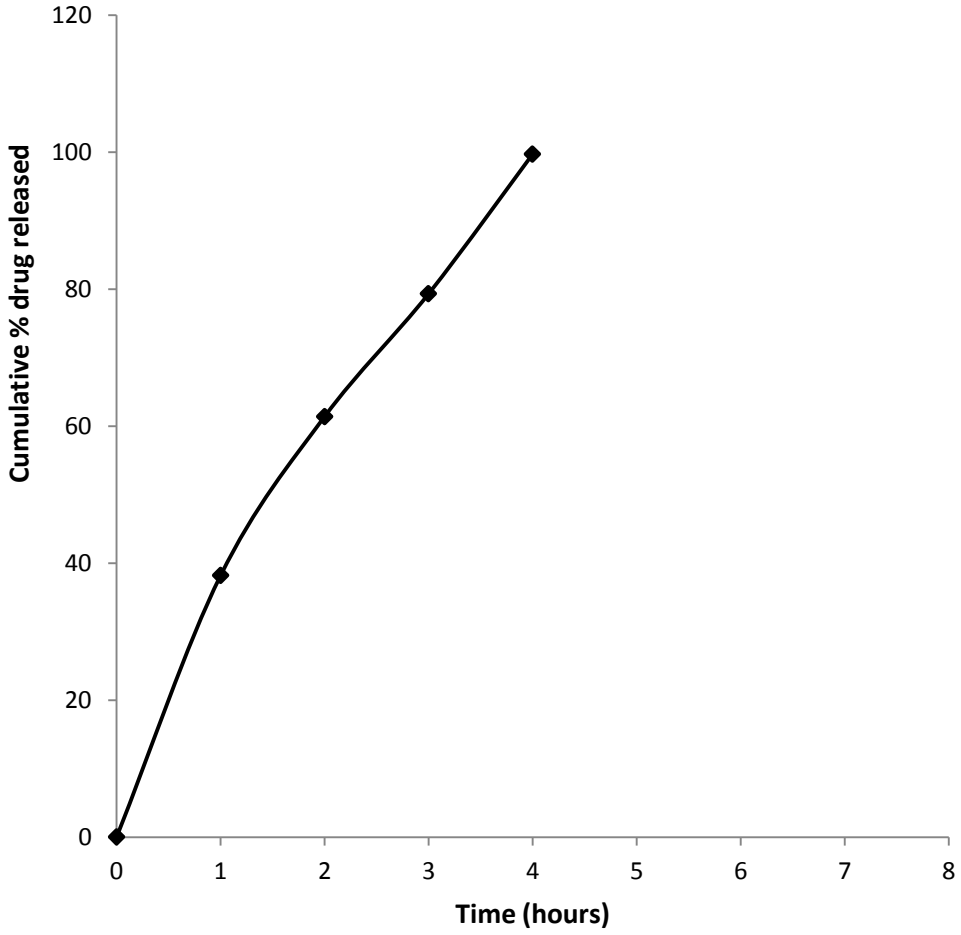


Figure 35: *In vitro* release profile of Levofloxacin hemihydrate loaded mucoadhesive microspheres (Formulation LM8)

(Bars represent mean of three values ± SD)

Table 16: *In vitro* release profile of Levofloxacin hemihydrate loaded mucoadhesive microspheres (Formulation LM9)

Time (hours)	Cumulative Percentage drug Released*	Standard Deviation**
0	0	0
1	31.92	1.25
2	49.17	0.89
3	59.41	2.20
4	67.17	2.57
5	78.75	2.95
6	85.77	1.73
7	94.46	0.84

*Each reading is an average of three determinations
**Standard deviation of three determinations

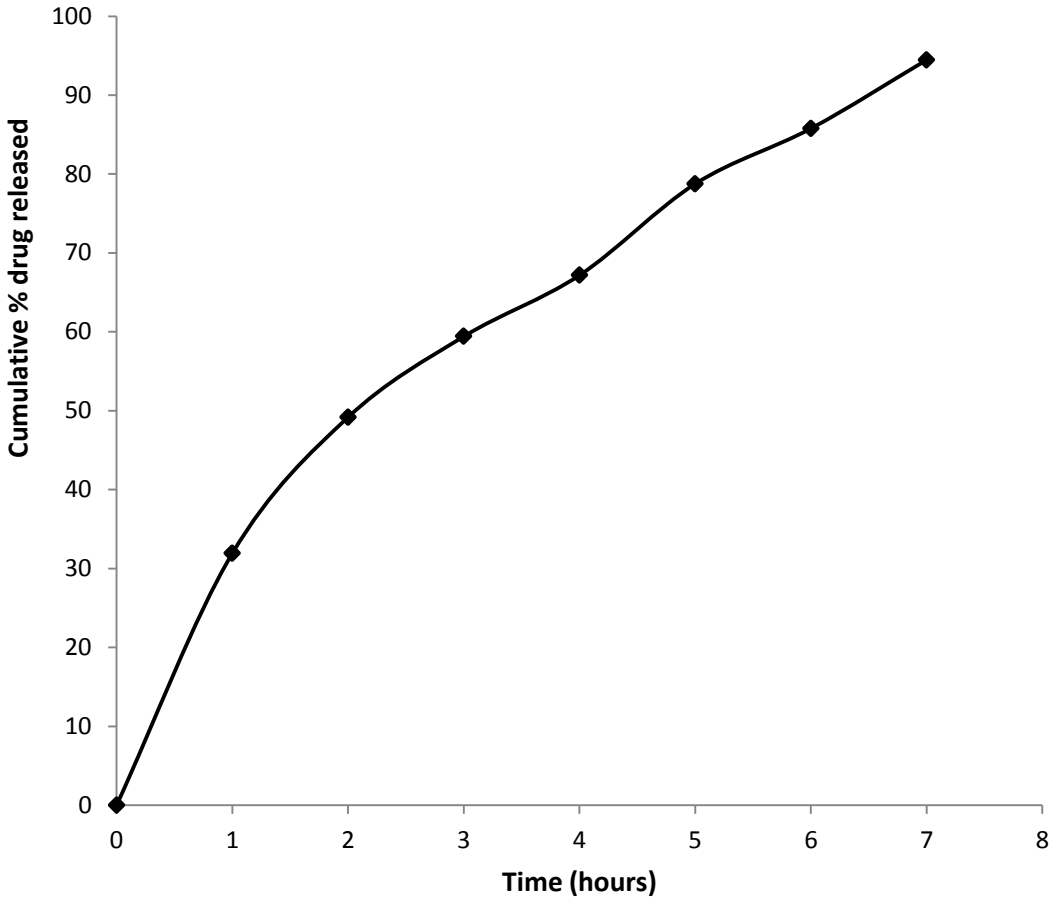


Figure 36: *In vitro* release profile of Levofloxacin hemihydrate loaded mucoadhesive microspheres (Formulation LM9)

(Bars represent mean of three values ± SD)

Table 17: *In vitro* release profile of levofloxacin hemihydrate loaded mucoadhesive microspheres (Formulation FA10)

Time (hours)	Cumulative Percentage drug Released*	Standard Deviation**
0	0	0
1	24.71	1.71
2	41.40	1.68
3	49.75	0.94
4	55.76	2.42
5	63.86	2.12
6	71.12	2.17
7	85.81	1.36

*Each reading is an average of three determinations
**Standard deviation of three determinations

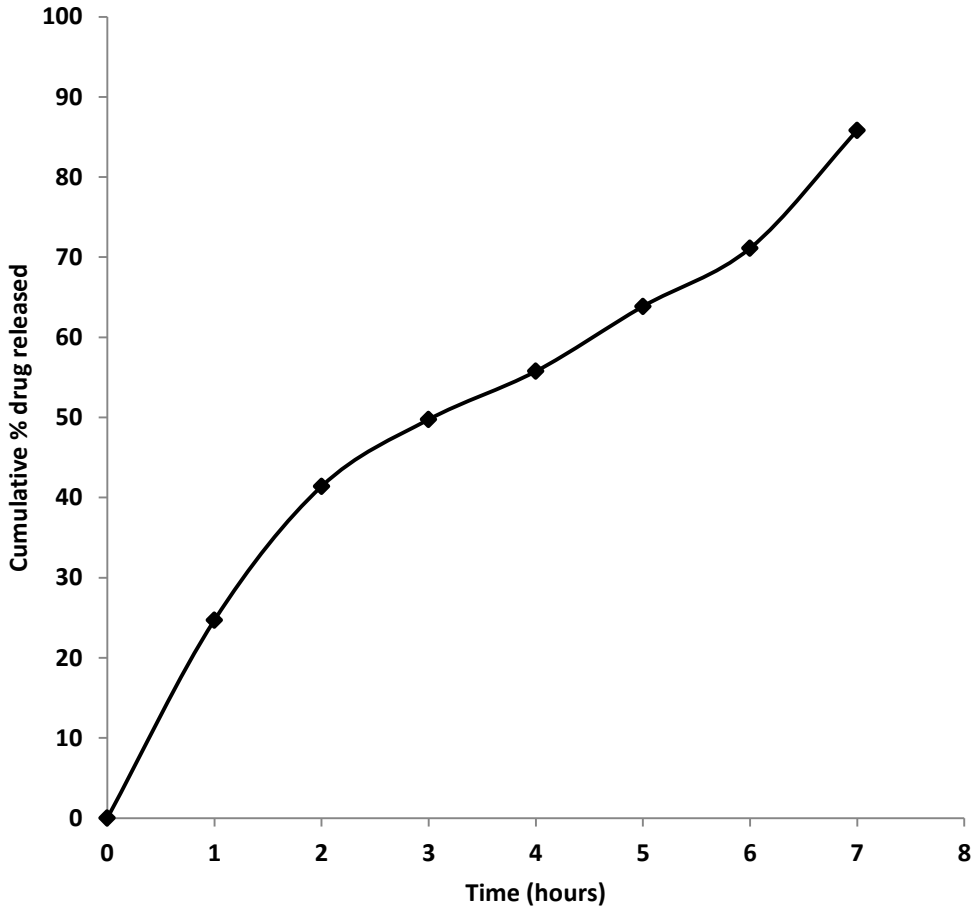


Figure 37: *In vitro* release profile of Levofloxacin hemihydrate loaded mucoadhesive microspheres (Formulation LM10)

(Bars represent mean of three values ± SD)

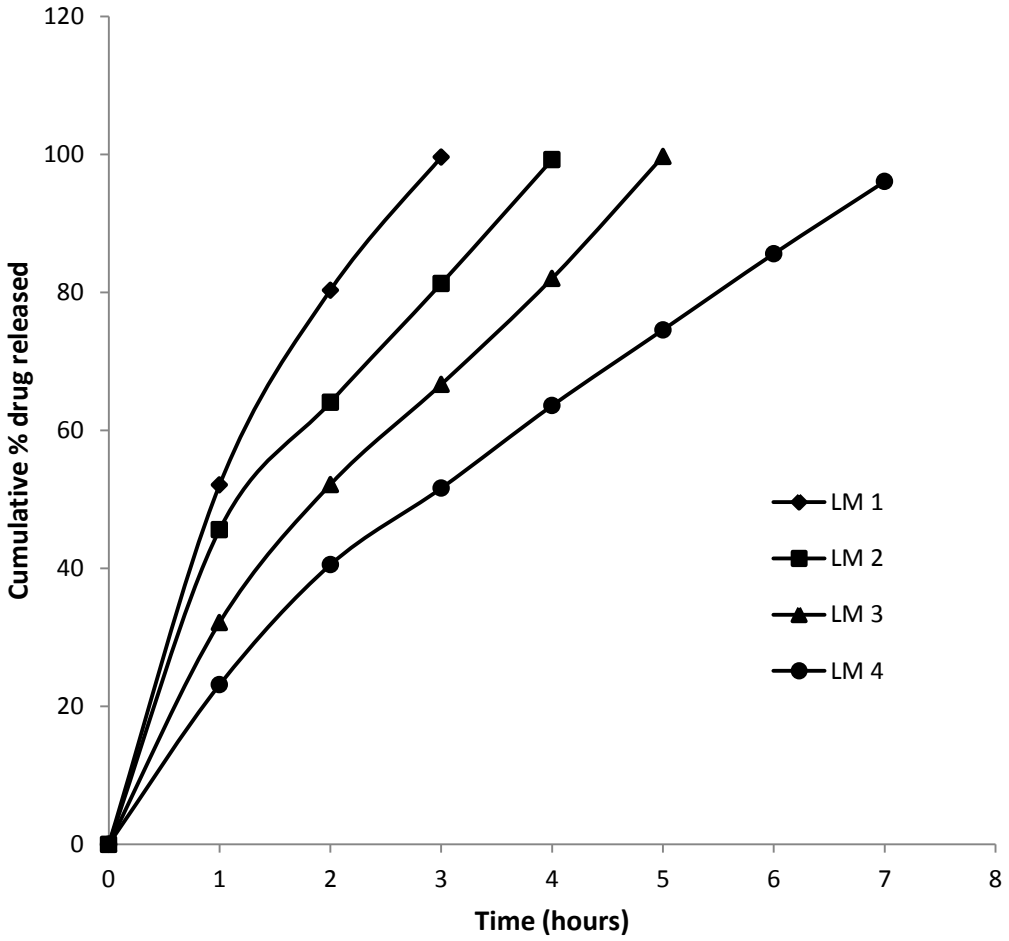


Figure 38: Effect of Eudragit RS 100 on the *in vitro* drug release characteristics of mucoadhesive microspheres of Levofloxacin hemihydrate in pH 1.2. (Bars represent mean of three values ± SD)

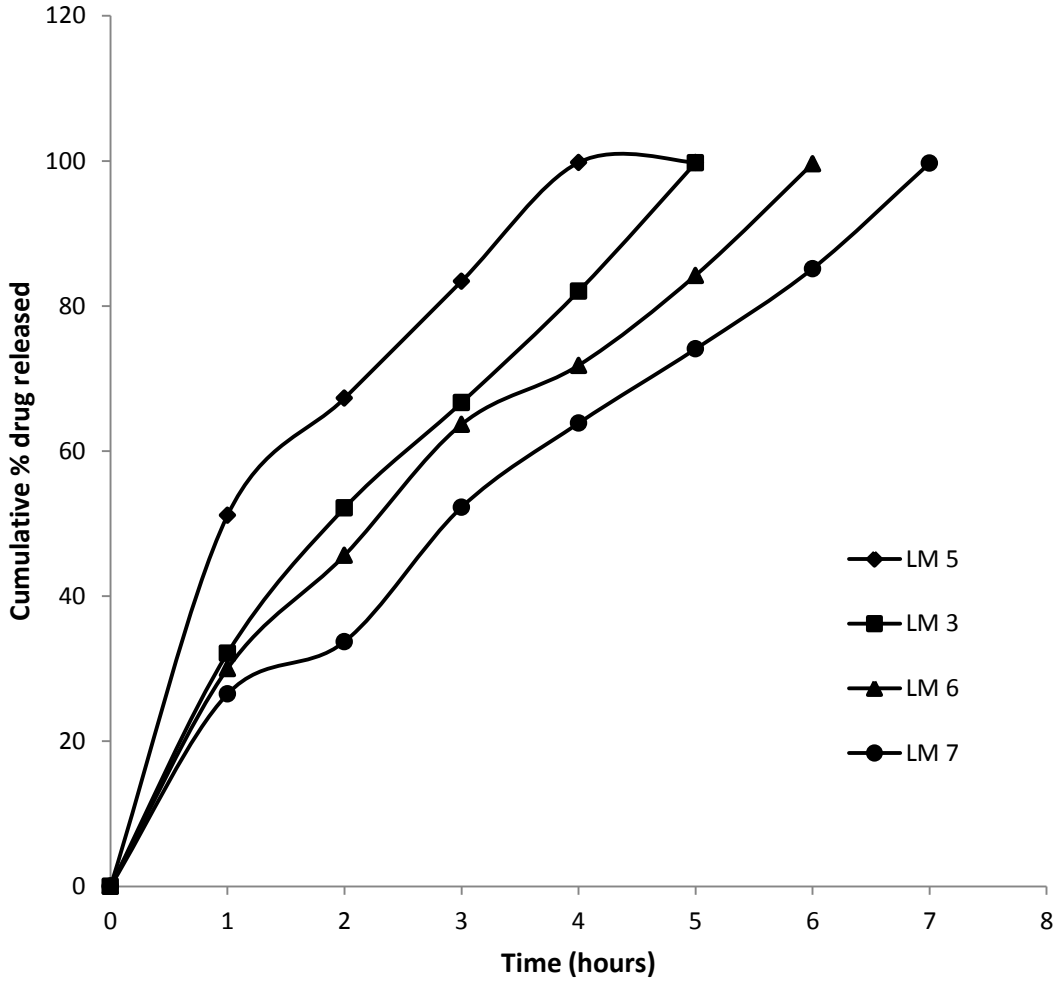


Figure 39: Effect of Carbopol 974P on the in *vitro* drug release characteristics of mucoadhesive microspheres of Levofloxacin hemihydrate in pH 1.2. (Bars represent mean of three values ± SD)

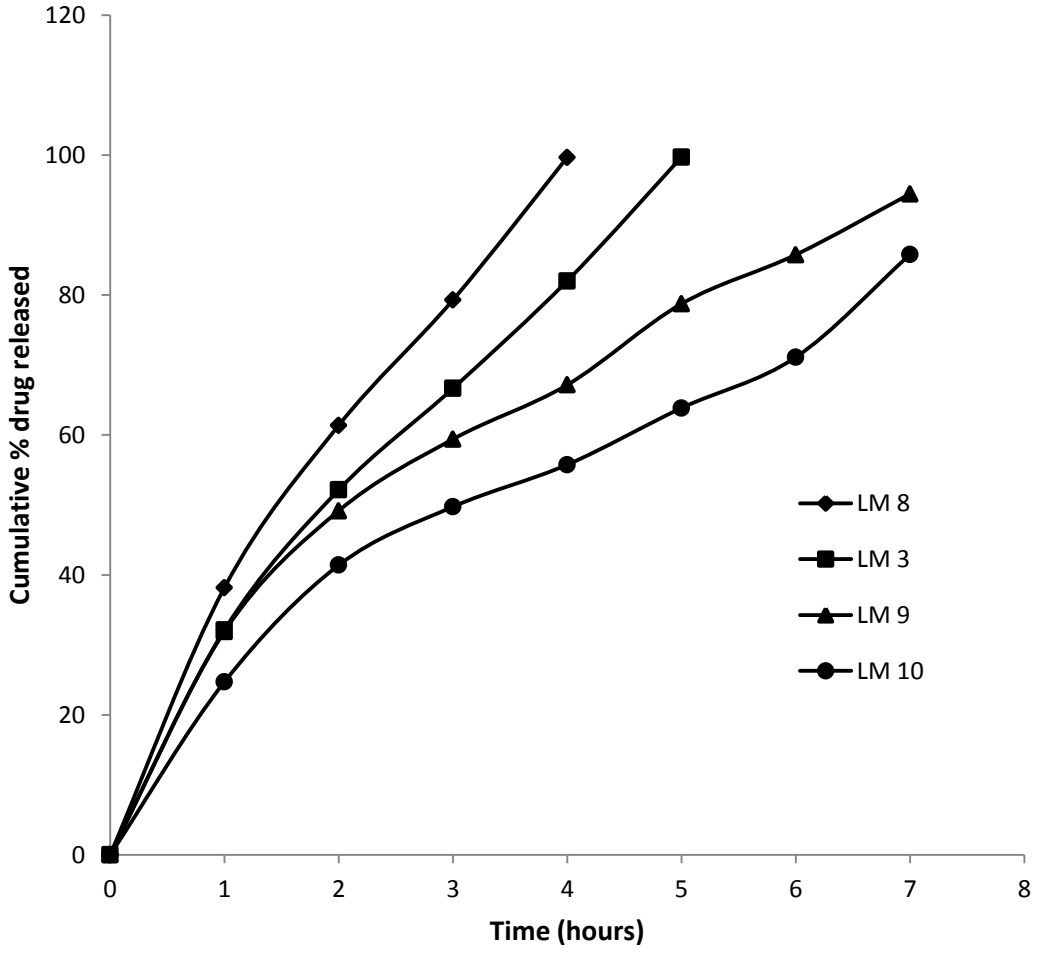


Figure 40: Effect HPMC K4M on the in *vitro* drug release characteristics of mucoadhesive microspheres of Levofloxacin hemihydrate in pH 1.2. (Bars represent mean of three values ± SD)

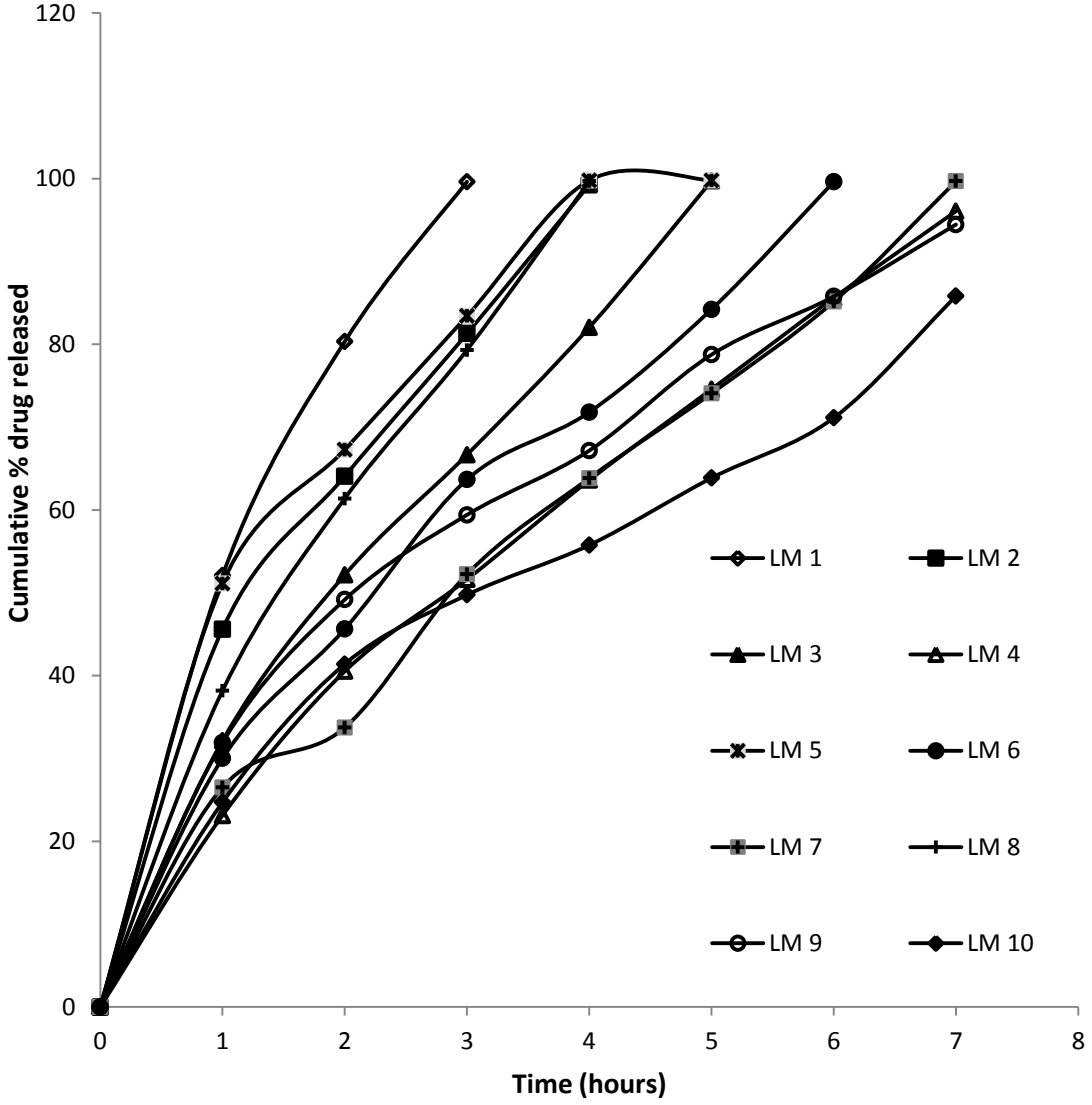


Figure 41: Comparativein *vitro* drug release characteristics of mucoadhesive microspheres of Levofloxacin hemihydrate in pH 1.2. (Bars represent mean of three values ± SD)

IN VITRO RELEASE KINETIC ANALYSIS

Table 18: *In vitro* release kinetic data of Levofloxacin hemihydrate loaded mucoadhesive microspheres

F Code	Zero order plot		First order plot		Higuchi plot	Korsemeyer peppa's plot	
	K_0	R^2	K_1	R^2	R^2	n	R^2
LM1	---*	---*	---*	---*	---*	---**	---**
LM2	18.9671	0.9954	-0.5729	0.8528	0.9978	---**	---**
LM3	16.5832	0.9986	-0.5338	0.7133	0.9901	---**	---**
LM4	12.9032	0.9951	-0.1819	0.9184	0.9942	0.6253	0.9998
LM5	17.3134	0.9978	-0.6965	0.7763	0.9928	---**	---**
LM6	14.2145	0.9982	-0.3821	0.7060	0.9933	0.5945	0.9991
LM7	13.4303	0.9964	-0.3052	0.7069	0.9943	0.6492	0.9988
LM8	19.6230	0.9953	-0.7128	0.7819	0.9923	---**	---**
LM9	11.3107	0.9849	-0.1613	0.9677	0.9989	0.5528	0.9983
LM10	09.8103	0.9919	-0.0954	0.9724	0.9945	0.5419	0.9975

* Insufficient data points to apply kinetics due to rapid release profiles
* *Insufficient data points to apply Korsmeyer-Peppas equation up to 70%.

K_0 – Zero order rate constant

K_1 – First order rate constant

R^2 – Regression coefficient

n- Diffusion exponent

ACCELERATED STABILITY STUDIES

Table 19: Accelerated stability data of Levofloxacin hemihydrate loaded mucoadhesive microspheres [Tested according to ICH Q1A(R2)]

S.No	Time (days)	Mucoadhesive strength (Mean ± SE) (n = 3)	Drug content (%) (Mean ± SD) (n = 3)	Drug release (%) (Mean ± SD) (n = 3)
1	Before storage (0 day)	75.47±0.23	25.85±0.34 (37.04)	99.62±0.23
2	30 days (After storage*)	75.01±0.67	25.82±0.93	99.46±0.21
3	90 days (After storage*)	74.66±0.86	25.78±0.51	99.28±0.18
4	180 days (After storage*)	74.33±0.43	27.60±0.68	98.83±0.66

*Storage at 40°C and 75% RH (n = 3).

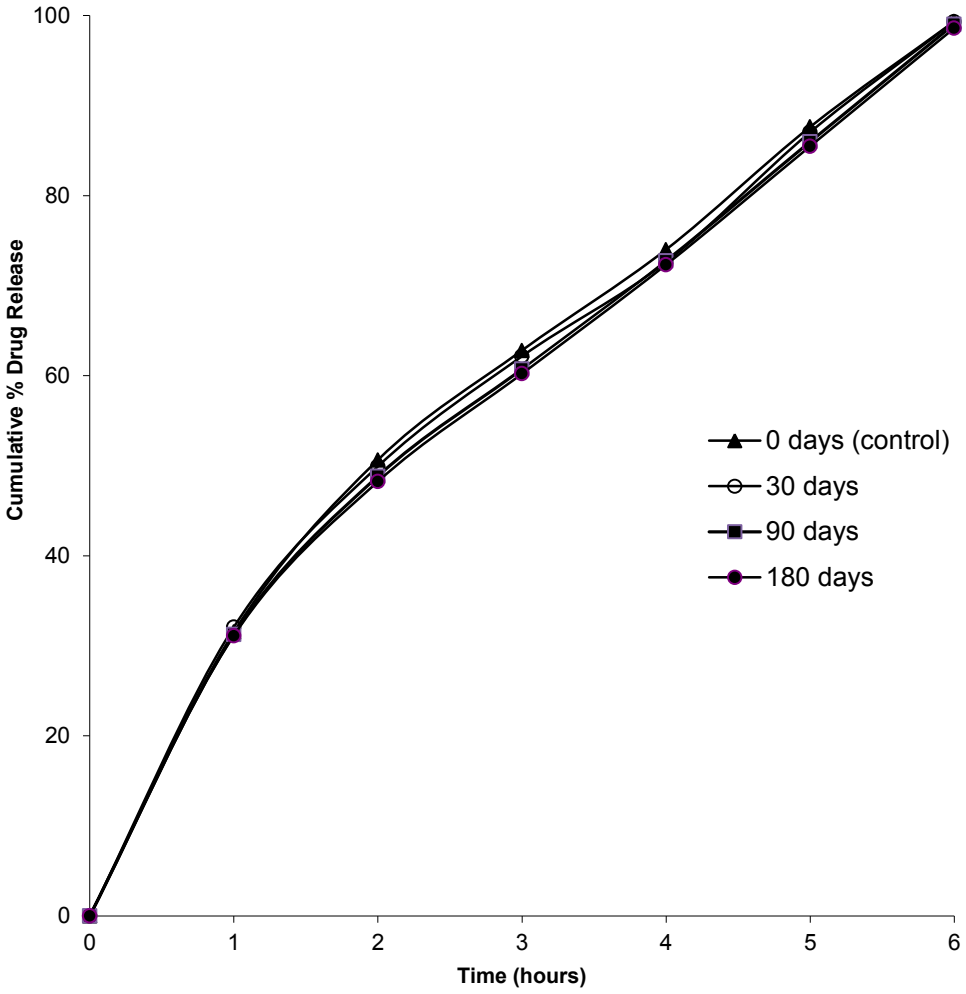


Figure 42: Stability study *in vitro* dissolution profile of Levofloxacin hemihydrate loaded microspheres in pH1.2 (Data points represents mean ± SD) (n=3).

9. DISCUSSION OF RESULTS

The mucoadhesive microsphere of levofloxacin hemihydrate was prepared by emulsification solvent evaporation technique. In this study, mucoadhesive polymers (HPMC K4M and carbopol 974P) were dispersed in matrix polymer (Eudragit RS 100.Studies had revealed that mucoadhesive microspheres adhere to the mucosal membrane more strongly when the mucoadhesive polymer was in dispersed state.[173]In this study, mucoadhesive polymers were dispersed in the microspheres in order to enhance mucoadhesion. Liquid paraffin and acetone were selected as outer and inner phases respectively. Acetone with a dielectric constant of 20.7 was used as disperse (inner) phase because solvents with dielectric constants between 10–40 show poor compatibility with liquid paraffin.[14,175]In order to optimize mucoadhesive and controlled release property, the various combinations of HPMC K4M, carbopol 974P and Eudragit RS 100 were used to prepare mucoadhesive microspheres [Table 1 & 2].

The standard curve of levofloxacin hemihydrate were plotted in 0.1 N HCl (pH 1.2) at 37^0C. It was found to be linear for the concentration ranges from 01 µgm/ml to 10 µgm/ml (R^2 =0.9961) for levofloxacin hemihydrate(Table 1& 25).

Percentage yield of microspheres

The percentage yield of levofloxacin hemihydrate loaded microspheres was shown in Table 2. The percentage yield for levofloxacin hemihydrate loaded microspheres were found to be in the range of 39.20±1.62% to 92.40±1.32% and 37.53±1.43% to 89.75±1.43% respectively. The microspheres yield increased with increase in the polymer concentration ($P< 0.05$).

Drug content and encapsulation efficiency

The effects of polymer concentration on the drug content and encapsulation efficiency of the prepared microspheres were shown in Table 3 &4. Drug content of the levofloxacin hemihydrate loaded microspheres varied from 24.56±0.41% to 29.64±0.42%. The encapsulation efficiency of the prepared microspheres varied from 52.31±1.16% to 86.33±0.67%. The encapsulation efficiency increased progressively by increasing the Eudragit RS100, HPMC K4M and carbopol 974P($P< 0.05$). High concentration of matrix polymer solution increases viscosity of the polymer droplet and delays the drug diffusion within the polymer droplets.[176]Larger microspheres have a smaller total surface area per unit mass and, consequently, a lower amount of drugs diffuses in to liquid paraffin. As a result drug content of the larger microspheres was higher.[177]

Particle size analysis- Shape and surface characterization

Viscosity of polymer solution is one of the most important factor related to formulation of microspheres.[178] The polymer (Eudragit RS 100) concentrations of 2%, 4%, 6% and 8% w/v were selected to determine optimum concentration of matrix polymer and also to study the effect of matrix polymer on drug release. Flake formation was observed when Eudragit RS 100 concentration was used at 2 %, 4%w/v, whereas maximum sphericity was observed at the 6 % w/v. Non-spherical microspheres were found when polymer concentration was used at 8% w/v. Therefore, 6% w/v of Eudragit RS 100 in acetone was found to be the optimum concentration for the polymer solution. The mean diameter of levofloxacin hemihydrate loaded microspheres were found to be in the range of 141.17±10.38µm to 543.91±18.94µm [Table 5]. The mean particle size increased with increasing concentration of Eudragit RS100, HPMC K4M carbopol 974P.

The reason for the increased particle size with the higher polymer concentration is that the viscosity of the dispersed phase. Viscosity of dispersed phase increases with increasing polymer concentration. When the viscosity of the dispersed phase increases, it will be more difficult to break up the droplets to a smaller size and larger droplet will be formed. Stirring efficiency was also reduced at higher polymer concentrations due to increased viscosity of the medium, resulting in an increase in the particle size.[179]If the amount

of polymer in each droplet if insufficient, the microspheres formed would not have enough strength to withstand the stirring, so it forms flakes. These factors might be the reason for changing particle size.

In vitro evaluation of mucoadhesiveness

The *in vitro* mucoadhesiveness study revealed that all the batches of prepared microspheres had good mucoadhesive property. Mucoadhesive property of the prepared microspheres varied from 53.55±0.57% to 86.54±0.81%.[Table 6]. The mucoadhesive property slightly increased with increasing the Eudragit RS 100 concentration. It may be due to the mucoadhesive nature of Eudragit RS 100 polymer.[180]A proportional increase in mucoadhesive strength of the formulation was observed with increase in the ratio of carbopol 974P and HPMC K4M. It was observed that mucoadhesive effect of HPMC K4M was less than carbopol 974P ($P< 0.05$). It may be due to strong mucoadhesive nature of carbopol 974P than HPMC K4M at pH 1.2.In thisd study mucoadhesiveness was evaluated at pH 1.2. The lower degree of ionization of carboxyl groups of carbopol 974P in this acidic pH results stronger interaction with mucus. These effects were reflected in this study.

Compatibility studies

DSC and FT-IR were performed on the raw materials and on the microspheres to detect interactions between the drug and the excipients.

FTIR studies

FTIR spectra were recorded for pure drug, drug-loaded microspheres and blank microspheres. According to the FTIR analysis results, excipients and active substances did not interact. The FTIR spectra of pure levofloxacin hemihydrate showed characteristic peaks for –COOH monomeric stretching and bonding at 3269 and 1045 cm^{-1}, alkanes $–CH_3$ and aromatic rings 2846 and 1618 cm^{-1},C=Ostretching vibration of the COOH group1721 cm^{-1}andC– F835 cm^{-1}. All theabove peaks peaks of levofloxacin hemihydrate were also present in FTIR spectrum of drug-loaded microspheres with slight broadening and reduction in intensity in drug-loaded formulations that confirm the presence of drug in the polymer without anyinteraction.

Differential Scanning Calorimeter (DSC) studies

DSC spectra were recorded for pure drug, drug-loaded microspheres and blank microspheres. The endothermic peak of levofloxacin hemihydrateis observed at about 237.3 °C [Figure 20]. Blank microspheres did not show any endothermic peaks because of

amorphous nature of Eudragit RS 100, HPMC K4M and carbopol 974P[Figure 21]. No endothermic peak corresponding to the levofloxacin hemihydrate was observed in levofloxacin hemihydrate loaded microspheres [Figure 22]. The absence of detectable crystalline domains in the microspheres clearly indicates that the drug was molecularly dispersed in the microspheres.The absence of detectable crystalline domains in the microspheres clearly indicates that drug was molecularly dispersed in the microspheres.

X-ray diffraction (XRD) studies

In order to investigate the physical nature of the encapsulated drug, the powder X-ray diffraction technique was used. Diffraction patterns of Eudragit RS 100, HPMC K4M and Carbopol 974P, levofloxacin hemihydrate and drug loaded microsphere formulation were studied.

The powder XRD patterns of pure levofloxacin showed characteristic peaks, indicating that they are in a crystalline state. Eudragit RS 100, HPMC K4M and carbopol 974P showed patterns of amorphous substances [Figure 24-26]. The XRD patterns of the levofloxacin hemihydrate loaded microspheres were completely different from those of pure drug and showed no characteristic peaks [Figure 27]. This demonstrates that the drug was in an amorphous state in the microspheres. These results were in good agreement with

DSC studies. The loss of drug diffraction peaks in microspheres indicates a change in their crystal form during the process.

In vitro dissolution studies

Dissolution test is a frequently used quality control method to evaluate drug release from oral dosage forms. Figure 28-37 shows the drug release profiles of levofloxacin hemihydrate loaded microspheres of various formulations.

An initial burst effect was observed in all the batches of microsphere formulations, which may be due to the drug being adsorbed or located near the surface of the microspheres. However, as the polymer swelling proceeds, the remaining drug was released at a slower rate. This bi-phasic pattern of drug release was a distinguishing feature of matrix diffusion systems.[181]The initial burst release effect was noticeably reduced with increase in polymer concentration.

A significant decrease in the rate and extent of drug release was observed with the increase in polymer concentration microspheres). This might be because of increase in the density of the polymer matrix and also an increase in the diffusional path length, which the drug molecules have to traverse.Increasing concentration of the Eudragit RS 100, drug release was decreased it might be due to increase ofthickness of gel diffusion layer.

Drug release was retarded by increasing the proportion of carbopol 974P and HPMC K4M respectively. HPMC K4M significantly influenced the drug release than carbopol 974P (P< 0.05). This may be due to gelling nature of carbopol 974Pin 0.1 N HCl. Because of its nonionic nature HPMC K4M swellingwas not affected by pH variation. At pH 1.2, HPMC K4M may form viscous gel around the microspheres and control the drug release.

***0In vitro* drug release and kinetics of release**

When the release data of levofloxacin hemihydrate loaded microspheres were plotted according to the first order equation, the formulations showed a fairly good linearity, with a R^2 value of 0.7133-0.9677[Table 18], whereas the same data, when plotted according to the zero order equation, improved the R^2 value of 0.9839-0.9984 and 0.9849-0.9986 [Table 18]. In our experiment, the *in vitro* release profiles of levofloxacin hemihydrate from all the formulations could at best be expressed by Higuchi's equation,[236] as the plots showed good linearity with R^2 value of 0.9901-0.9989 [Table 18]. Good linearity was observed with the zero order equation. The slope of the regression line from the Higuchi plot indicates the rate of drug release and thus confirmed that the mode of release was diffusion, and to further confirm the diffusion mechanism, the data were fit into the Korsmeyer[237]*et al.,* equation which showed high linearity with a comparatively high slope (n) value of 0.5419-0.5945for levofloxacin

hemihydrate loaded microspheres [Table 18]. This n-value indicated a coupling of diffusion and erosion mechanism. This type of drug release is called as anomalous diffusion. This indicates that drug release from the microspheres follows a non-Fickian trend as reported earlier.[182]The presence of swelling polymers within the matrix structure might be responsible for the drug release controlled by more than one process. Thus, with regard to release kinetics, the data best fits in the Higuchi model and showed zero order release with a non-Fickian diffusion mechanism. The results showed that when an appropriate blend of these polymers was used, the drug release became more uniform in its kinetic approach towards zero order.

Selection of best formulation

The normal mucus turnover rate is 4–6 hours in rats [183-185,186]and likely similar values in humans.[187]The mucus turnover rather than the mucus-polymer interaction that controls the presenceof mucoadhesive formulations through theGIT.[188]Based on the mucus turnover rate and dissolution time, formulations LM 6 (formulations consisting of 6 % w/v Eudragit RS, 1.5 % w/v carbopol 974P and 1% w/v HPMC K4M) were selected as best formulations. Accelerated stability studies LM 6.

Accelerated stability testing according to ICH Q1A (R2)

The optimized formulation (FA6 & FC6) were stored in a stability chamber (Remi CHM- 10 S®, India) at 40 ± 2°C and at a humidity of 75 ± 5% RH for 6 months and observed for the drug content, mucoadhesiveness and *in vitro* drug release on 0, 30, 90, and 180 days [Table 19]. The zero time samples were used as controls. No remarkable changes were observed in drug content, mucoadhesiveness and *in vitro* drug release in stability studies [Figure 42].

10. CONCLUSION

The aim of the present research work was to formulate and evaluate mucoadhesive microspheres of levofloxacin hemihydrate using the mucoadhesive polymers (carbopol 974P and HPMC K4M for *H. pylori* eradication. These two polymers (carbopol 974P and HPMC K4M) act as complimentary to each other in that carbopol 974P increases the mucoadhesion and HPMC K4M other hand helps to control drug release. From all of the experiments performed, it can be concluded that the developed mucoadhesive polymers can be successful in the effective for treatment of *H. pylori* infection. The developed mucoadhesive polymers may decrease the short comings of conventional drug delivery systems. It can also deliver the antimicrobial effect to the infected mucosal area. It is possible that mucoadhesive polymers with uniform gastric distribution can targetthe *H. pylori*-infected sites more effectively and could optimize antibiotic monotherapy of *H. pylori*, which could be of definite therapeutic benefit. Further more, inorder to confirm the efficiency of developed formulations, *In-vivo* buoyancy and pharmacokinetic studies are necessary.

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